STUDIES ON CAROTENOID PRODUCTION

IN CULTURES OF

BIXA ORELLANA

by

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DECLARATION

I hereby declare that this thesis was composed by myself and the work described herein to be my own, except where indicated otherwise.

Margaret G. Boyd

Edinburgh, 1991

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Margaret G. Boyd, 1991

ABBREVIATIONS

λ_{max}	absorption maximum
ATP	adenosine triphosphate
6-BAP	6-benzylaminopurine
°C	degrees centigrade
ca.	approximately
CAT	chloroamphenicol acetyltransferase
CHS	chalcone synthase
cm.	centimetre(s)
conc.	concentrated
CPW13M	medium containing CPW salts with 13% mannitol
d.	day(s)
2,4-D	2,4-dichlorophenoxyacetic acid
DMAPP	dimethylallyl pyrophosphate
DNA	deoxyribonucleic acid
eg.	for example
et al.	*et alia*
Fig.	figure
FPP	farnesyl pyrophosphate
FW	fresh weight
g.	gram(s)
xg.	x gravitational force
GGPP	geranylgeranyl pyrophosphate
GPP	geranyl pyrophosphate
h.	hour(s)
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HPLC	high performance liquid chromatography
ie.	that is
2-IP	2-isopentenyl adenine
IPP	isopentenyl pyrophosphate
kg.	kilogram(s)
KIN	kinetin
l.	litre(s)
m.	metre(s)
M	molar
µl.	microlitre(s)
µm.	micrometre(s)
µmol.	micromole(s)
mg.	milligram(s)
min.	minute(s)
ml.	millilitre(s)
mm.	millimetre(s)
MRGR	mean relative growth rate
MS	Murashige and Skoog
MS-N	MS medium lacking nitrogen
MS50%NAA	MS medium with 50% less NAA than control
MS-NAA	MS medium lacking NAA
MS-NP	MS medium lacking nitrogen and phosphate
MS-P	MS medium lacking phosphate
MS1%S	MS medium with 1% sucrose
MS6%S	MS medium with 6% sucrose

MS8%S	MS medium with 8% sucrose
MS12%S	MS medium with 12% sucrose
MS3%S+M	MS medium with 3% sucrose and mannitol (51.5g.l.$^{-1}$)
MS8%S-N	MS medium with 8% sucrose and lacking nitrogen
MVA	mevalonic acid
i-N	inorganic nitrogen
NAA	naphthaleneacetic acid
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nm.	nanometre(s)
no.	number
O.D.	optical density
OM	original medium
osmol.	osmole(s)
i-P	inorganic phosphate
P	probability
PAL	phenylalanine ammonia-lyase
PCV	packed cell volume
%	percent
pH	negative log of the hydrogen ion concentration
psi.	pounds per square inch
R_f	retention factor
rpm.	revolutions per minute
s.e.	standard error
sec.	second(s)
SH	Schenk and Hildebrandt
T	time
TDC	tryptophan decarboxylase
TLC	thin layer chromatography
v/v	volume per volume
λ	wavelength
W	weight
w/v	weight per volume
ZEA	zeatin
<	less than

CONTENTS

ABSTRACT

The aim of this project was to study the production of carotenoid pigments in cultured cells of *Bixa orellana* to develop cultures with a high yield of product.

Orange/red pigmented cells were detected in phloem and cortical tissues of stems, petioles and roots of *B.orellana* and in the phloem and spongy mesophyll of leaves. Bixin, β-carotene, lutein and several unidentified pigments were present in the vegetative tissues whereas only bixin was present in the seeds.

Both callus and suspension cultures of *B.orellana* were established. The pigment levels in the callus cultures were very low and appeared to decline as the cultures aged. Attempts to increase pigment production in callus using standard procedures such as reduction of auxin, removal of nitrogen or phosphate, addition of picloram and cell selection methods were not successful. However, pigmented cells were detected in newly initiated callus from root explants and the number of these cells increased as the callus was established. Manipulating the culture medium using 8% instead of 3% sucrose or by removing nitrogen and/or phosphate decreased the growth of newly initiated callus tissue and increased the pigment on a per mg. fresh weight basis compared to the control.

Carotenoid production in *B.orellana* suspension cultures was shown to occur in the lag and early growth phases of batch growth. Depletion of nitrogen or an increase in the sucrose concentration from 3% to 8% resulted in an increase in pigment accumulated in suspension cultures. An increase in the sucrose concentration in the culture medium delayed cell division and growth of cultures and, within limits, the higher the sucrose concentration the longer the delay before an increase in cell number could be detected. There appeared to be a positive correlation between sucrose concentration and pigment production and the increases in pigment were shown to be at least partly due to the increase in the number of pigmented cells. Zeaxanthin and β-carotene were detected in suspension cultures but not bixin. Raising the level of sucrose increased the number and the amount of pigments present and this was shown to be primarily due to the osmolality of the medium. From this it would appear that the increased pigment levels in cultures grown in the presence of high sucrose concentrations was due primarily to the osmotic property of the medium. In the discussion the relationship between carotenoid production, growth and differentiation is explored.

Chapter 1

Introduction

1.1 THE ANNATTO FOOD COLOUR (E160b)

1.1.1 *Bixa orellana* as a Source of Food Colouring

Bixa orellana L. is a large shrub or small tree, 2-5m. high, which is native to tropical America (Ingram and Francis, 1969). It belongs to the family *Bixaceae*, however, the position of the plant at the ordinal level is still under debate (see Decraene, 1989). The seeds of this plant are coated with a thin pulpy resinous layer which is orange/red coloured. It is from this layer that a commercial dye, which is used mainly as a food colouring, is obtained. Due to this commercial value the plant is now grown in many tropical countries. The main producers are: Bolivia, Brazil, Ceylon, Dominican Republic, Ecuador, Guyana, India, Jamaica, Mexico and Peru (Ingram and Francis, 1969; Preston and Rickard, 1980).

The orange/red pigment obtained from the seeds of *B.orellana* is named annatto (E160b). Its chief application is in colouring dairy products and margarine but it is also used with many other foods. Some of the foods in which annatto is employed are listed in Table 1.1. and the use of annatto as a food colour is discussed by Timberlake and Henry (1986), Francis (1987), Knewstubb and Henry (1988). Annatto is generally used alone, however, it can be mixed with the orange/red paprika oleoresin extracted from the fruits of *Capsicum annum* to give redder shades.

1.1.2 The Composition of Annatto

Several methods are available for extracting the colour from seeds of *B.orellana*. The FAO/WHO (1982) have defined specifications for the methods of production of annatto extracts. Annatto extract in edible oil may be obtained by pretreatment of the seeds with steam or hot water followed by extraction with vegetable oil. Solvent extraction is also used. Aqueous annatto extracts are prepared by heating the seeds, or the solvent extract of the seeds, with a solution of sodium hydroxide at 70°C. or lower, followed by boiling and filtration. Extraction methods for annatto are described in detail by Ingram and Francis (1969), Preston and Rickard (1980), Bhalkar and Dubash (1983), Tong (1984) and Chao *et al.* (1991).

Thin layer chromatography (TLC), paper chromatography and high performance liquid chromatography (HPLC) have all been used to identify the compounds in annatto (M^{c}Keown, 1961; Francis, 1965; Dendy, 1966; Reith and Gielen, 1971; Tirimanna, 1981; Rouseff, 1988; Chr. Hansen's Laboratory Ltd., Copenhagen, Denmark). The major pigment of the seeds is the carotenoid cis-bixin, $C_{25}H_{30}O_4$

Table 1.1

Foods in which annatto food colouring can be used (as listed by Chr. Hansen's Laboratory Ltd., Copenhagen, Denmark).

Bearnaise sauce	Lard
Biscuit filling	Liqueur
Butter	Liquorice Allsorts
Cake filling	Macaroni
Cereals	Margarine
Cheese	Mayonnaise
Cheese powder	Milk powder
Cheese snacks	Pastries
Cheese spread	Pizza filling
Chocolate filling	Popcorn
Confectionery	Puddings
Crackers	Pudding powder
Cream dessert	Salad oil
Cream/Custard powder	Sausages
Dressing (oil)	Sausage skin
Drinking mix powder	Shortening
Fish	Snacks
Fruit wine	Soft drinks
Hollandaise sauce	Soup powder
Ice cream	Tart filling
Ice lollies	Yoghurt

(McKeown, 1961). Cis-bixin is orange in colour and insoluble in vegetable oil. On heating, it is converted to the more stable isomer, trans-bixin, which is red and soluble in oil. Some degradation products, notably a C_{17} pigment, are also formed (McKeown and Mark, 1962; McKeown, 1963, 1965; Preston and Rickard, 1980). The structure of bixin shows it has a free and an esterified carboxyl group as end groups (see Fig. 1.1). Upon saponification the methyl ester group is split off resulting in the diacid norbixin, $C_{24}H_{28}O_4$ (see Fig 1.1), which is water soluble (Tong, 1984). Oil soluble annatto is used in dairy and fat-based products (eg. butter, margarine, processed cheese) and the water soluble form in cheese, sugar confectionery and baked goods.

1.2 BIOTECHNOLOGY AS A SOURCE OF FOOD COLOURINGS

1.2.1 The Use of Food Colourings

The addition of colour to foods extends back in time to before the existence of written records (Klaui and Bauernfeind, 1981). It is generally believed that the use of added colours has a direct bearing on the acceptance of many foods. Indeed colour is an early signal of the inherent qualities of food such as freshness and readiness for consumption. (see Goldenberg, 1977; Klaui and Bauernfeind, 1981). Today many foods are processed in some way before they reach the consumer and colours are added to foods for a number of reasons (see Goldenberg, 1977; Timberlake and Henry, 1986):

(1) To make food more attractive and therefore appetizing.

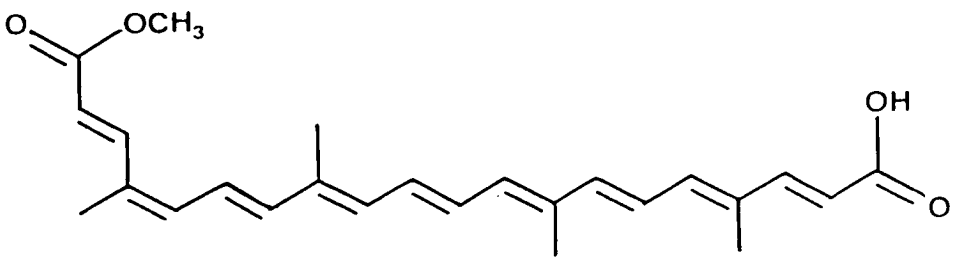
(2) To ensure uniformity of colour of different batches of a product.

(3) To replace natural colours that are lost during food processing eg. by high temperatures.

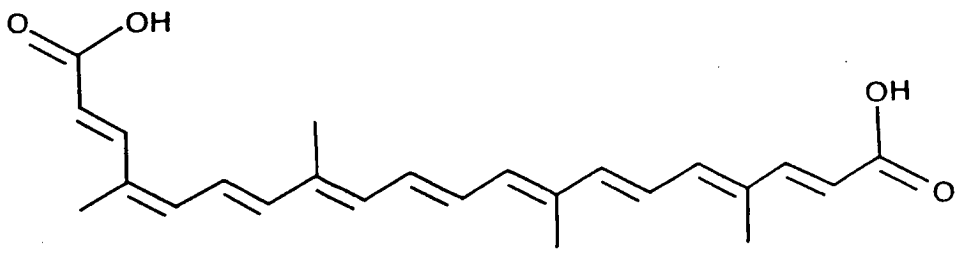
Food colourings are not needed in the same technological sense as preservatives or antioxidants and the vast majority of our foods would still have the same quality and flavour without added colours (Goldenberg, 1977). However, in general, the consumer desires attractively coloured foods so it follows that the food industry will continue to acquire and use a wide array of acceptable, safe food colourings to satisfy consumer preferences (Bauernfeind, 1981).

Figure 1.1

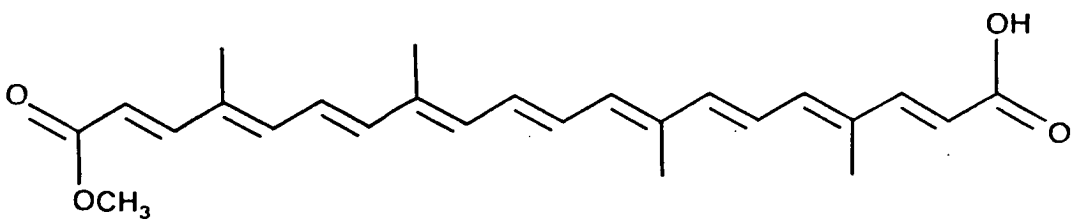
Structures of cis (labile form) and trans (stable form) isomers of bixin and norbixin.



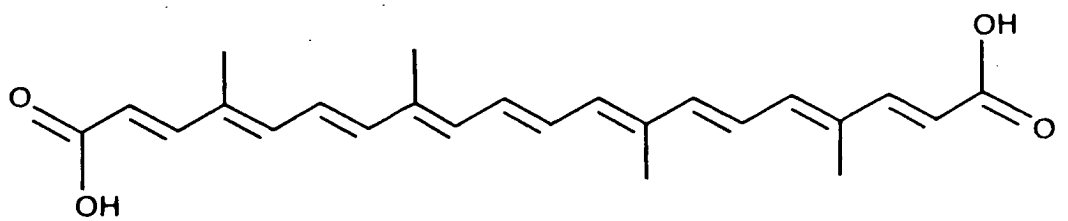
cis-bixin ($C_{25}H_{30}O_4$)



cis-norbixin ($C_{24}H_{28}O_4$)



trans-bixin ($C_{25}H_{30}O_4$)



trans-norbixin ($C_{24}H_{28}O_4$)

1.2.2 Problems with Food Colours

Today there is a great deal of public concern over the possible adverse health effects of certain artificially produced food additives, especially colours. In the past synthetic dyes have been largely used because of their stability in foods and ease of use. However, many of these are suspected to be toxic and in some countries have been banned. The red dye Amaranth (E123), together with Sunset Yellow (E110) and the widely used yellow dye Tartrazine (E102) have been associated with allergies. In particular, tartrazine is known to cause asthma and is implicated in hyperactivity, especially in children (see Timberlake and Henry, 1986). This has led to a demand for fewer additives and for naturally produced food additives to be used in food. A 'natural colour' can be defined as a colour extracted from a plant or animal in such a way that the pigments remain chemically unchanged (Timberlake and Henry, 1986).

Plant pigments are ideal for use as food colourings since they are essentially the same as those contained in the original plant material and have a long history of consumption over thousands of years. However, despite this history of use, some plant pigments are not permitted as food additives because their toxicity has not been tested and so they are not considered safe. The plant pigments currently in use as natural food colourings include flavonoids (largely anthocyanins), carotenoids, chlorophylls, betalaines and others (curcumin and carthamin). These natural food colours are discussed in detail by Bauernfeind (1981), Klaui and Bauernfeind (1981), Timberlake and Henry (1986), Francis (1987), Knewstubb and Henry (1988).

1.2.3 The Potential of Biotechnology for Food Colouring Production

Many of the plants from which food colourings are obtained grow in tropical and sub-tropical regions of the world. This can cause problems since the cost and availability of these colourings can be affected by the economy and political situation in the countries of origin. There are also problems with uncontrolled exploitation, disappearance of habitat and difficulties in cultivating the plants. As a result of these problems the production of useful compounds, such as food colourings, by plant tissue culture has become increasingly significant in the field of biotechnology and is considered as a viable alternative. The use of biotechnology methods would allow natural products to be produced. The potential of biotechnology for the production of colours for the food industry is discussed in detail by Bell and White (1989).

1.3 CAROTENOID PRODUCTION IN HIGHER PLANTS

1.3.1 General Pathway for Carotenoid Production

The majority of carotenoids of higher plants are C_{40} tetraterpenes biosynthesized by the well-established isoprenoid pathway as shown in Fig. 1.2 (Britton, 1988). In the main pathway of mevalonic acid (MVA) biosynthesis, acetyl-CoA and acetoacetyl-CoA condense to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA is then reduced to form MVA. Acetyl-CoA is normally synthesized either from pyruvate by the action of pyruvate dehygrogenase, pyruvate itself being formed by glycolysis, or by β-oxidation of fatty acids. The early stages of the pathway are considered to be identical in the biosynthesis of all terpenoids. In carotenoid biosynthesis the terpenoid chain is built up to the C_{20} level and two C_{20} units condense to give the typical C_{40} carotenoid skeleton. Thus it is only the stages after geranylgeranyl pyrophosphate that are unique to the formation of carotenoids.

A summary of the stages of carotenoid biosynthesis is shown in Fig. 1.3. This pathway is a general one for carotenoid synthesis and it does not include the detailed synthesis of all carotenoids. The pathway for bixin synthesis is not known, but it is thought to occur by oxidative degradation of C_{40} carotenoids (see Zechmeister, 1962; Jondiko and Pattenden, 1989). In support of this proposal the carbon skeleton of bixin is identical with the middle section of the β-carotene molecule but the chromophore is terminated by conjugated carboxyl groups. Although such circumstantial evidence supports the proposal of a degradative pathway no detailed evidence for bixin has been published (Jondiko and Pattenden, 1989).

1.3.2 Location of Carotenoids

Carotenoids accumulate in the chloroplasts of all green leaves and it has been found that the leaves of higher plants usually contain the same carotenoids : β-carotene, lutein, violaxanthin and neoxanthin are the major ones (Goodwin, 1980; Goodwin and Britton, 1988). Pigments which appear frequently, but not constantly, in smaller amounts are α-carotene, β-cryptoxanthin, zeaxanthin and antheraxanthin (Goodwin, 1976). Leaf carotenoids are specifically located in the grana of chloroplasts in the form of pigment-protein complexes.

Carotenoids are also located in chromoplasts, carotenoid-containing plastids, especially in non-photosynthetic tissues such as flowers, fruits and roots. In many cases the chromoplasts develop from chloroplasts eg. in flowers and ripening fruit,

Figure 1.2

Summary of the pathway of isoprenoid biosynthesis (see Britton, 1988).

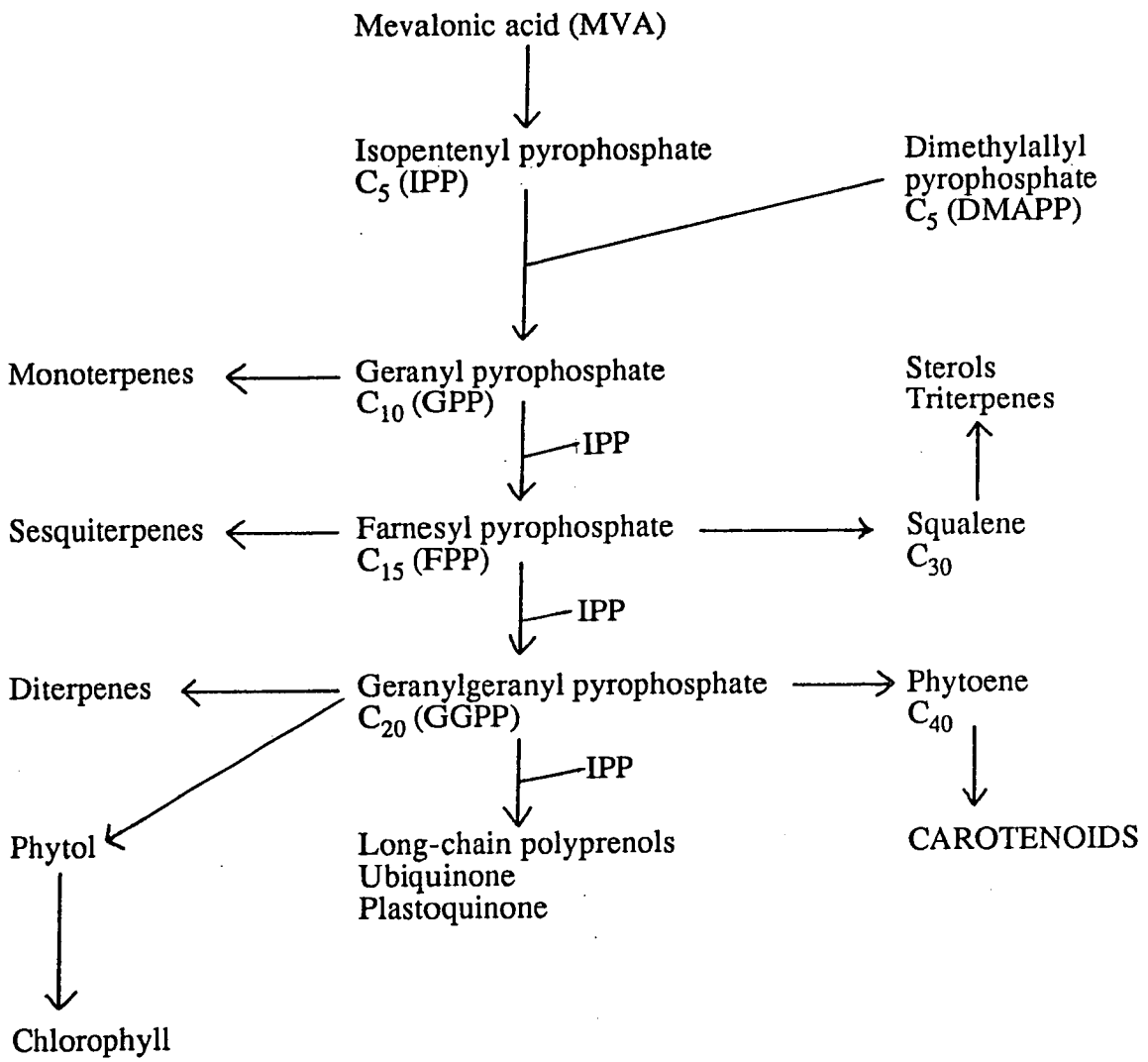
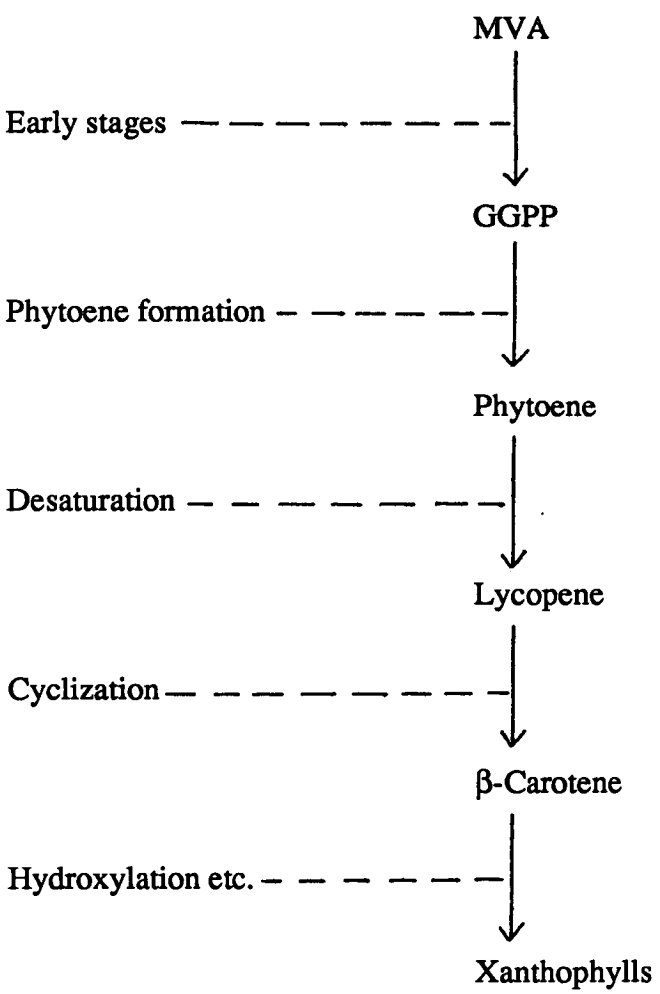


Figure 1.3

Summary of the stages of carotenoid biosynthesis (see Britton, 1988).



but they can also develop from proplastids and amyloplasts (Kirk and Tilney-Bassett, 1978; Goodwin, 1976, 1980; Thomson and Whatley, 1980).

1.3.3 Function of Carotenoids

In chloroplasts carotenoids are essential for the survival of the photosynthetic organism and they have two major functions in photosynthesis. They act as accessory light-harvesting pigments and as photoprotective agents, preventing photoxidative damage (see Siefermann-Harms, 1987; Cogdell, 1988). The first function allows photosynthetic organisms to utilize light over a wide spectral range. The second of these two functions is essential because without carotenoids there would be no photosynthesis in the presence of oxygen.

No physiological function is known for carotenoids in chromoplasts (Thomson and Whatley, 1980). Possibly the main function is to attract animals to the bright colours especially in fruit and flower chromoplasts. In fruit the animals are likely to eat the fruit and hence distribute the seeds. In flowers attraction of insects will allow cross-pollination to occur. It is difficult to think of a function for root chromoplasts. In the case of carrots it is possibly a mutation that has been selected out and propagated by man since wild carrot is white and does not contain chromoplasts (see Kirk and Tilney-Bassett, 1978).

1.4 THE PRODUCTION OF SECONDARY PRODUCTS BY PLANT TISSUE CULTURE

1.4.1 Secondary Metabolism in Cultured Plant Cells

Man is dependant on plants for maintenance of the biosphere, for food and for medicines. Higher plants are able to synthesize a complex array of chemical structures of which secondary metabolites are one group of commercially important compounds (Fowler, 1987). A number of secondary metabolites have properties which are of social and commercial benefit to man. For example, plant secondary metabolites are used in the pharmaceutical, fragrance, flavouring and food industries (see Collin, 1987; Fowler, 1987; Phillipson, 1990; Wink, 1990). Such fine chemicals can be extracted directly from the plant but there are difficulties in obtaining these plants as they tend to grow under tropical conditions (Collin, 1987). Some compounds that were once obtained from the whole plant are now synthesized chemically (Fowler, 1987). However, there are problems with the chemical synthesis of secondary metabolites. For example, it may not be possible to synthesize the

compound chemically. Many secondary metabolites are chiral molecules and this can make synthesis difficult or impossible (Wink, 1990). Therefore, chemical synthesis is not cost effective and biological production of the compounds is usually a more economic alternative. Another factor that favours biological synthesis of secondary metabolites is that natural products are required, especially in the food industry. Therefore, despite difficulties in obtaining chemicals directly from the plant, they are still the major source of many secondary metabolites. Indeed, in the USA higher plants are the source material for 25% of the active ingredients in prescriptions (Fowler *et al.*, 1990; Phillipson, 1990).

In order to ensure a constant supply of designated secondary metabolites, plant biotechnologists have investigated plant cell cultures as a possible source of these valuable compounds (see Collin, 1987; Fowler, 1987; Wink, 1990). Three major ways in which plant cell cultures may be used to provide natural products are (see Fowler, 1987; Fowler *et al.*, 1990):

(1) As an alternative source of established products.

(2) As a source of enzymes as possible aids for biotransformation systems.

(3) As a source of novel products.

However, despite a long and intensive period of research the number of commercial successes has been limited and only two compounds, shikonin and berberine, are being produced commercially from cultures of *Lithospermum erythrorhizon* and *Coptis japonica* respectively (Fujita, 1988). A problem with plant tissue culture is that in many instances the yields of secondary metabolites obtained are much lower than those in the plants from which they were derived and the spectrum of compounds produced is often quite different to that in the plant (see Fowler, 1983, 1986; Collin, 1987; Yeoman *et al.*, 1990). There are some reports of cultures accumulating secondary metabolites at levels equalling and occasionally exceeding those observed in the plant (Fowler, 1986). However, in general, low product yields are a major barrier to the commercial production of compounds by plant tissue culture.

1.4.2 Relationship Between Growth and Secondary Metabolism

Evidence in the literature suggests that secondary metabolism in cultured plant cells is related to the growth of the cultures (see Lindsey and Yeoman, 1985; Collin,

1987; Komamine *et al.*, 1989; Yeoman *et al.*, 1989). In a number of instances it has been shown that accumulation occurs in parallel with growth whereas others show accumulation occurs after growth has slowed or stopped. The production of most secondary metabolites has been found to be inversely related to growth (Yeoman *et al.*, 1980, 1982). Here accumulation tends to be associated with the downturn in culture growth and with biochemical and structural differentiation (Lindsey and Yeoman, 1985; Collin, 1987). For example, Lindsey and Yeoman (1983) showed that in a number of solanaceous species organization and alkaloid accumulation occurred at the end of the growth phase. Also maximum accumulation of anthocyanin in *Vitis* sp. suspension cultures (Hirose *et al.*, 1990) and betaxanthin in *Portulaca grandiflora* cells (Bohm *et al.*, 1991) took place during the stationary phase of growth. There are, however, some cases where secondary metabolites are produced during growth eg. the accumulation of podophyllotoxin in callus tissue of *Podophyllum peltatum* (Kadkade, 1982) and the accumulation of betacyanin in suspension cultures of *Phytolacca americana* (Sakuta *et al.*, 1986; Hirose *et al.*, 1990). Here, there is a positive correlation between secondary metabolism and culture growth. Reports have also been made of accumulation occurring during the lag phase of growth, a period after subculture when there is no growth or cell division as the cultures adapt to their new environment. For example, production of germichrysone in cell cultures of *Cassia torosa* (Noguchi and Sankawa, 1982), accumulation of phenolics in *Nicotiana tabacum* suspension cultures (Cvikrova *et al.*, 1988) and accumulation of ferruginol in *Salvia miltiorrhiza* cell cultures (Miyasaka *et al.*, 1985). This production may be an extension of the accumulation associated with the downturn of growth since there is no growth in the initial period after subculture. Thus it would appear that there are at least two patterns of secondary metabolism accumulation in relation to growth and differentiation in cultured plant cells.

1.4.3 The Metabolic Switch from Primary to Secondary Metabolism

The inverse relationship between secondary metabolism and growth of cultures, which occurs frequently, has been explained on the basis of a switch from primary to secondary metabolism. It has been argued that the pathways of primary and secondary metabolism compete for common precursors depending on whether culture conditions permit rapid or slow growth (Phillips and Henshaw, 1977; Lindsey and Yeoman, 1983, 1985; Yeoman, 1987). Thus if cells are dividing slowly precursors appear to be diverted from primary to secondary metabolism. The evidence of Lindsey and Yeoman (1984), Lindsey (1985, 1986) and Hall and Yeoman (1991) further support this competition theory. Alterations in enzyme/substrate

compartmentalization (Lindsey and Yeoman, 1985) or changes in enzyme activities (Yeoman *et al.*, 1980) must be involved in this regulation. The explanation can also be extended to take into account that organized cultures, which grow more slowly and so might be expected to have limited primary metabolism, accumulate higher yields of secondary metabolites. This implies that the interrelationship between cell organization and growth rate is important in that slow growth associated with organization allows a diversion of precursors between metabolic pathways (Yeoman, 1987). It is possible that cell organization may affect metabolism by changing the ultrastructure of the cells and cell contact may be essential for exchange of nutrients (Lindsey and Yeoman, 1983; Collin, 1987).

1.5 ATTEMPTS TO INCREASE THE YIELDS OF SECONDARY PRODUCTS IN TISSUE CULTURE

1.5.1 Methods of Increasing Secondary Metabolite Production

Product yield is probably the most important factor of any biologically based process (Fowler, 1986). The yield has to be large enough to ensure a product at a marketable price is obtained or to make the process more competitive than existing commercial systems. Due to the low yields of secondary metabolites which generally occur in tissue culture a great deal of work has been devoted to increasing the yields of these compounds (see Mantell and Smith, 1983; Collin, 1987). Three approaches have been used to try to increase the yield of product from cell cultures.

(1) The selection of high-yielding strains.

(2) The manipulation of the physical or chemical environment of the cultured cells so that the balance between growth and differentiation is altered.

(3) The manipulation of the genome using genetic engineering methods.

1.5.2 Selection of High-Yielding Strains

In this approach cells which produce the desired product are isolated and used to start new cultures. The cells of interest can arise through natural variation which is present within a culture or variation can be induced by treatments such as ultraviolet light which induce mutation. The techniques of selection of cell lines from callus, suspension cultures and protoplasts are now fully established and form an important part of plant cell technology which is currently being exploited to improve the yields of secondary metabolites and improve plant (crop) productivity (Collin and Dix,

1990). A problem with this method is that the cultures tend to be unstable which means the production of a designated product falls with time.

1.5.3 Manipulation of the Physical or Chemical Environment

In this approach the cultures are generally manipulated so that the biosynthetic pathways leading to the substances concerned are expressed and operative. The manipulations used include altering the concentration of minerals in the culture medium such as nitrogen and phosphorus (Knobloch and Berlin, 1980, 1981; Lindsey, 1985), varying the concentration of the carbon source (Bhatt *et al.*, 1983; Do and Cormier, 1991a), changing the concentration and balance of growth regulators (see Mantell and Smith, 1983; Collin, 1987), altering the pH of the medium (see Mantell and Smith, 1983), changing the temperature and light conditions (see Mantell and Smith, 1983; Collin, 1987), supplying precursor(s) to the product (Brodelius and Nilsson, 1980; Lindsey and Yeoman, 1984) and the addition of biotic and abiotic elicitors (Holden *et al.*, 1988a, b; Eilert, 1989; Brodelius, 1990).

In many culture systems it is often the highly aggregated cultures which accumulate the most secondary metabolites. It is because of this that the method of immobilizing plant cells in an inert matrix has been developed (Lindsey *et al.*, 1983; Lindsey and Yeoman, 1984, 1986). As a result of immobilization, cells are encouraged to grow together in a multicellular, partially organized state and the chemical conditions around the cells can be changed rapidly. Immobilizing cells raises the possibility of creating conditions in which secondary metabolites can be produced continuously over long periods provided the culture remains viable and the metabolite is released into the culture medium.

A combination of selecting for high-yielding strains and manipulating the environment has been used on *Lithospermum erythrorhizon* and *Coptis japonica* cultures and the yields that were obtained have enabled the commercial production of shikonin and berberine respectively to take place.

1.5.4 Manipulation of the Genome Using Genetic Engineering Methods

Although yields of secondary metabolites have been increased by selecting for high-yielding clones and manipulating the environment little is known about how metabolism is regulated. Central to the problem of low yields is a basic understanding of how metabolism is regulated eg. what enzymes and therefore genes are involved. If a particular enzyme in a pathway is shown to be regulatory it may be

possible to clone the gene for the enzyme and amplify or modify its activity. Such a gene, if placed in cultured cells, could then eventually lead to an increase in secondary metabolite production. Recent advances in recombinant DNA technology now provide the means to clone genes and introduce them into plant cells.

The regulation of plant natural product synthesis at the biochemical, molecular and genetic levels is best understood in the case of phenylpropanoid biosynthesis (see Dixon and Lamb, 1990). A number of studies have investigated phenylpropanoids, both their synthesis and function as gene regulators. In one study a phenylalanine ammonia-lyase (PAL) gene promoter fragment [PAL 2] from bean (*Phaseolus vulgaris*) was transferred to tobacco by *Agrobacterium tumefaciens* and it was found that the PAL 2 promoter could transduce a complex set of developmental and environmental cues into an integrated program of gene expression to regulate the synthesis of phenylpropanoids (Liang *et al.*, 1989). In a further study Elkind *et al.* (1990) showed that phenylpropanoid biosynthesis in tobacco was perturbed when a PAL gene from bean which had been modified by inclusion of cauliflower mosaic virus 35S enhancer sequences in its promoter was introduced. Thus the transgene disrupted PAL regulation. Loake *et al.* (1991) have also suggested that phenylpropanoid compounds may act as natural and specific regulators of plant gene expression. They found that a chimeric gene construct containing a bean chalcone synthase (CHS) promoter fused to the chloroamphenicol acetyltransferase (CAT) reporter gene was strongly expressed when put into alfalfa protoplasts that were then exposed to a fungal elicitor and the expression was regulated by phenylpropanoids.

However, studies have not only been carried out on phenylpropanoids. Songstad *et al.* (1990, 1991) found that the protoalkaloids tryptamine and tyramine were accumulated at greater levels in tobacco when a DNA clone encoding tryptophan decarboxylase (TDC) from *Catharanthus roseus* driven by the CaMV 35S promoter was introduced.

These examples demonstrate the potential of using recombinant DNA technology to produce high-yielding cell lines for a particular secondary metabolite by interfering with or enhancing regulatory pathways. Other approaches involving genetic manipulation could also prove useful in raising yields of secondary metabolites. One possibility is the use of antisense genes to block the formation of an enzyme which could degrade the desired product or block the activity of enzymes which compete for the added precursor (see Mol *et al.*, 1990). Other possibilities are that multiple copies of regulatory genes could be cloned and inserted or the promoter sequence(s) of the

gene(s) for secondary metabolism could be modified so that the gene(s) would always be active and the product continuously synthesized (see Yeoman *et al.*, 1990).

Such genetic engineering methods may in the future allow plant biotechnologists to produce secondary metabolites by plant cell cultures at yields which enable commercial production on a wider scale than at present.

1.6 AIMS AND OBJECTIVES

The aim of this project was to study the production of carotenoid pigments in cultures of *Bixa orellana* as a means to increasing product yield. The aim encompasses the following objectives:

(1) To investigate the nature and distribution of the pigments in the plant and to compare them with those in tissue cultures.

(2) To establish both callus and suspension cultures.

(3) To select for and improve pigmented (carotenoid producing) cultures.

(4) To determine the fate of the carotenoid pigment during callus initiation and subsequently in culture.

(5) To determine the effect of culture conditions on carotenoid production.

Chapter 2

Materials and Methods

2.1 PLANT MATERIAL

Plants of *Bixa orellana* L. (family Bixaceae) were raised from seed (Chr. Hansen's Laboratory Ltd., Reading) in 7.5cm. pots in Levington's compost (Fison's Ltd., U.K.). The plants were grown in a controlled environment chamber at 25°C. ± 2°C., 16h. day, illuminance 135μmol.m.$^{-2}$sec.$^{-1}$ (Thorn warmwhite fluorescent, Phillips tungsten). Watering was carried out daily and the plants were repotted when required.

2.2 TISSUE AND CELL CULTURE

2.2.1 Media Preparation

2.2.1.1 Growth regulator stock solutions

Stock solutions of naphthaleneacetic acid (NAA), 2-isopentenyl adenine (2-IP), zeatin (ZEA), kinetin (KIN) and 6-benzylaminopurine (6-BAP) (all obtained from Sigma Chemical Company, Poole, Dorset) were prepared by dissolving a known amount in 3ml. M KOH (BDH Ltd., Poole, Dorset). The solution was then added slowly to 50ml. distilled water. After thorough mixing the total volume was made up to 100ml. with distilled water in a volumetric flask. All of these stock solutions were stored at 4°C.

2.2.1.2 Standard growth medium

The medium used routinely as the standard growth medium for callus and suspension cultures was a supplemented Murashige and Skoog medium (MS) (Murashige and Skoog, 1962). The constituents of this medium are listed in Table 2.2.1. To prepare the medium, commercial Murashige and Skoog medium (Imperial Laboratories Ltd., Andover, Hants) was used at a concentration of 4.71g.l.$^{-1}$. This was dissolved in distilled water, then sucrose (BDH Ltd., Poole, Dorset) and growth regulators were added. Unless otherwise stated in the text, the sucrose concentration added to MS medium was 30g.l.$^{-1}$ and the growth regulators used were: 0.2mg.l.$^{-1}$ 2-IP (1 x 10^{-6}M) and 1mg.l.$^{-1}$ NAA (5.4 x 10^{-6}M). The pH was adjusted to 5.8 using M KOH or M HCl then the medium was made up to the required volume with distilled water.

In addition to the above constituents solid media used for callus cultures contained 10g.l.$^{-1}$ agar (Oxoid No.1, Oxoid Ltd., Basingstoke, Hampshire).

2.2.1.3 Schenk and Hildebrandt medium and White's medium

Both Schenk and Hildebrandt medium (SH) (Schenk and Hildebrandt, 1972) and White's medium (White's) (White, 1963) were prepared from powdered mixtures supplied by Imperial Laboratories Ltd., Andover, Hants. The constituents of each of the media can be found in Table 2.2.1. The media were prepared by dissolving the appropriate amount of powder (4.46g.l.$^{-1}$ SH and 1.31g.l.$^{-1}$ White's) in distilled water along with 30g.l.$^{-1}$ sucrose. Growth regulators (0.1mg.l.$^{-1}$ 2-IP (5 x 10^{-7}M) and 0.5mg.l.$^{-1}$ NAA (2.7 x 10^{-6}M)) were added and the pH was adjusted to 5.8. Finally, the media were made up to the correct volume with distilled water and agar (10g.l.$^{-1}$) was added.

2.2.1.4 Seed germination medium

Sterile seeds were germinated on medium containing MS (4.71g.l.$^{-1}$), sucrose (30g.l.$^{-1}$) and agar (10g.l.$^{-1}$). The pH of the medium was adjusted to 5.8 using M KOH or M HCL.

2.2.1.5 MS media lacking nitrogen, phosphate and nitrogen and phosphate

MS media lacking nitrogen (MS-N), phosphate (MS-P) and nitrogen and phosphate (MS-NP) were used in the experiments described in sections 3.3, 3.4 and 3.5. The constituents of these media are listed in Table 2.2.2. MS medium lacking KH_2PO_4, KNO_3 and NH_4NO_3 was made up at ten times the normal concentration and stored at -40°C. in 100ml. portions. When required, the concentrated stock medium was diluted with distilled water, then KH_2PO_4, KNO_3, and NH_4NO_3 were added as required. A calculated amount of KCl was also added to restore the potassium level in the medium. Sucrose and growth regulators were added then the pH was adjusted to 5.8 before the media were made up to the required volume. Solid media also contained agar (10g.l.$^{-1}$).

Table 2.2.1

The composition of Murashige and Skoog medium (MS), Schenk and Hildebrandt medium (SH) and White's medium (White's).

Constituents	Concentration in Media ($mg.l.^{-1}$)		
	(MS)	(SH)	(White's)
$CaCl_2.2H_2O$	440	200	-
$Ca(NO_3)_2$	-	-	208.5
$CoCl_2.6H_2O$	0.025	0.1	-
$CuSO_4.5H_2O$	0.025	0.2	0.001
FeNaEDTA	36.7	-	4.59
$FeSO_4.7H_2O$	-	15	-
H_3BO_3	6.2	5.0	1.5
KCl	-	-	65
KH_2PO_4	170	-	-
KI	0.83	1.0	0.75
KNO_3	1900	2500	80
$MgSO_4.7H_2O$	370	400	720
$MnSO_4.4H_2O$	22.3	13.2	7.0
MoO_3	-	-	0.0001
Na_2EDTA	-	20	-
$NaH_2PO_4.2H_2O$	-	-	18.7
$Na_2MoO_4.2H_2O$	0.25	0.10	-
Na_2SO_4	-	-	200
$NH_4H_2PO_4$	-	300	-
NH_4NO_3	1650	-	-
$ZnSO_4.7H_2O$	8.6	1.0	3.0
i-inositol	100	1000	-
nicotinic acid	0.5	5.0	0.5
thiamine HCl	0.1	5.0	0.1
pyridoxine HCl	0.5	0.5	0.1
glycine	2.0	-	3.0
2-isopentenyladenine (2-IP)	0.2 *	0.1	0.1
naphthaleneacetic acid (NAA)	1.0 *	0.5	0.5
sucrose	30,000	30,000	30,000
pH	5.8	5.8	5.8

* Concentration of 2-IP and NAA unless otherwise stated in the text.

Table 2.2.2

The composition of Murashige and Skoog medium lacking nitrogen (MS-N), phosphate (MS-P) and nitrogen and phosphate (MS-NP).

Constituents	Concentration in Media ($mg.l.^{-1}$)		
	(MS-N)	(MS-P)	(MS-NP)
$CaCl_2.2H_2O$	440	440	440
$CoCl_2.6H_2O$	0.025	0.025	0.025
$CuSO_4.5H_2O$	0.025	0.025	0.025
FeNaEDTA	36.7	36.7	36.7
H_3BO_3	6.2	6.2	6.2
KCl	1403	93	1496
KH_2PO_4	170	-	-
KI	0.83	0.83	0.83
KNO_3	-	1900	-
$MgSO_4.7H_2O$	370	370	370
$MnSO_4.4H_2O$	22.3	22.3	22.3
$NaMoO_4.2H_2O$	0.25	0.25	0.25
NH_4NO_3	-	1650	-
$ZnSO_4.7H_2O$	8.6	8.6	8.6
i-inositol	100	100	100
nicotinic acid	0.5	0.5	0.5
thiamine HCl	0.1	0.1	0.1
pyridoxine HCl	0.5	0.5	0.5
glycine	2.0	2.0	2.0
2-isopentenyladenine (2-IP)	0.2	0.2	0.2
naphthaleneacetic acid (NAA)	1.0	1.0	1.0
sucrose	30,000	30,000	30,000
pH	5.8	5.8	5.8

2.2.2 Sterilization Techniques

2.2.2.1 Sterilization by heat

All glassware, distilled water and nutrient media which did not contain heat labile substances were sealed with a double layer of aluminium foil and then were sterilized by autoclaving at 121°C. for 20min. at a steam pressure of 15 psi.

2.2.2.2 Sterilization with ethanol

All cell culture manipulations were carried out in a laminar air-flow cabinet. The surfaces of the cabinet were swabbed and sprayed with absolute ethanol before and after use. Any instruments used in culture manipulations were stored in ethanol and flamed immediately prior to use.

2.2.2.3 Sterilization with hypochlorite

All seed and plant material was surface sterilized in sodium hypochlorite before being used in tissue culture.

a. Sterilization of seeds

Seeds were initially stirred in chloroform for 3h. to remove the pigment. The seeds were then dried and subjected to a rapid pre-sterilization in 95% (v/v) ethanol for 30sec. followed by immersion in 20% (v/v) sodium hypochlorite (2-2.8% available chlorine) (A. & J. Beveridge Ltd., Edinburgh) in distilled water containing three drops of Tween-20 (Sigma Chemical Company, Poole, Dorset) for 15min. The seeds were then removed under sterile conditions and washed three times in sterile distilled water before being used.

b. Sterilization of plant tissue

All plant tissue was sterilized after excision from the plant and prior to inoculation onto medium. The cut ends were sealed with hot wax (Paraplast-Lancer, Eire). The tissue was then pre-sterilized in 70% (v/v) ethanol in distilled water for 30sec. followed by immersion in 15% (v/v) sodium hypochlorite (1.5-2.1% available chlorine) in distilled water containing three drops of Tween-20 for 20min. The tissue was then washed three times in sterile distilled water under sterile conditions prior to use.

2.2.3 Initiation and Maintenance of Cultures

2.2.3.1 Germination of sterile seeds

Sterilized seeds (*ca.* 25) were transferred to and spread out evenly over the surface of the germination medium contained in 9cm. polystyrene Petri dishes (Sterilin Ltd., Hounslow, U.K.). The Petri dishes were then sealed with parafilm (American National Can, Greenwich) and placed in the dark at 25°C. ± 2°C. for up to six weeks.

2.2.3.2 Culture conditions

All cell cultures were grown under the following conditions:

TEMPERATURE: 25°C. ± 2°C.

PHOTON FLUX DENSITY: 25μmol.m.$^{-2}$sec.$^{-1}$

LIGHT SOURCE: Compton warmwhite fluorescent

LIQUID CULTURE AGITATION: Continuous rotation in a horizontal plane, 98rpm., 8mm. amplitude.

2.2.3.3 Initiation of callus cultures

After sterilization, plant material was transferred to a sterile Petri dish (9cm.) in the laminar air flow cabinet. Stem and petiole tissue were cut into transverse sections (3-4mm.) using a scalpel and forceps. Also, discs (1cm.) of leaf tissue were cut from either side of the midrib using a flamed cork borer. Transverse sections of root tissue (4mm.) were cut from sterile seedlings obtained from seeds germinated under sterile conditions. These transverse sections and discs of tissue were placed on 15ml. solid medium in sterile 5cm. polystyrene Petri dishes. The Petri dishes were sealed with parafilm and left under standard culture conditions (2.2.3.2). After 4 weeks any callus formed was removed from the explants and subcultured onto fresh medium.

2.2.3.4 Initiation of suspension cultures

Unless otherwise stated in the text, suspension cultures were initiated by adding 1-2g. of friable callus to 250ml. or 100ml. conical (Erlenmeyer) flasks containing 50ml. or 20ml. liquid medium respectively. The flasks were covered with a double layer of sterile aluminium foil and placed on an orbital shaker (2.2.3.2). After 1 week, the cultures were filtered through a sterile 800μm. nylon sieve (Lockertek,

Warrington) and any lumps removed. The cells and medium which passed through the sieve were left for a further week on the orbital shaker before being subcultured.

2.2.3.5 Maintenance of callus and suspension cultures

Stock cultures were maintained on MS medium as callus and suspension cultures.

Callus cultures were subcultured every 2 weeks onto fresh solid medium in 9cm. Petri dishes.

Suspension cultures were also subcultured every 2 weeks. The cultures were filtered (64μm. sieve) (Lockertek, Warrington) and placed in a sterile Petri dish. 1-2g. wet weight of cells was transferred to fresh liquid medium using a perforated spoon spatula.

2.3 ANALYTICAL TECHNIQUES

2.3.1 Measurement of Culture Growth and Viability

2.3.1.1 Determination of fresh weight

The fresh weight of callus cultures was determined by weighing the Petri dish containing medium with and without cells and calculating the difference between the two weights.

The fresh weight of the suspended cells was determined after they had been filtered under vacuum through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, U.K.).

2.3.1.2 Determination of dry weight

To determine the dry weight a known weight of cells was dried overnight (*ca.* 16h.) in a hot air oven at 80°C., cooled in a desiccator and weighed.

2.3.1.3 Determination of cell number

In order to determine the cell number of suspension cultures, an initial digestion was necessary to loosen the cells from one another. A method adapted from that of Brown and Rickless (1949) was used.

To a known fresh weight of cells (*ca.* 50mg.), 0.5ml. of 10% chromic acid solution (w/v chromium trioxide in distilled water) was added. This mixture was left

overnight (*ca.* 16h.) at room temperature after which it was homogenized by pumping the solution in and out of a Pasteur pipette 10 times. The cell number was then estimated with a Hawksley crystalite haemocytometer (grid volume 1.8µl.). The solution was diluted to give a cell density of 100-200 cells $grid^{-1}$. Six grids were then counted, the mean of which was used to calculate the cell number.

2.3.1.4 Determination of packed cell volume

Suspension cultures (20ml.) were poured into 50ml. graduated centrifuge tubes (J. Bibby Science Products Ltd., Stone, Staffordshire) and centrifuged for 5min. at a force of 1000xg. The packed cell volume (PCV) was calculated as the percentage cell volume of the total culture volume. That is,

$$PCV = \frac{\text{volume of cell mass}}{\text{total culture volume}} \times 100$$

2.3.1.5 Determination of cell viability

The viability of cells was determined using the method of Widholm (1972). This method is based on the ability of cells to cleave fluorescein diacetate molecules to produce free fluorescein which fluoresces under ultraviolet light.

A stock solution of fluorescein diacetate, 0.5% w/v fluorescein diacetate (Sigma) in acetone (BDH), was prepared. Prior to analysis the stock solution was added dropwise to 5ml. of culture medium until the medium became slightly turbid. One drop of culture was then mixed with one drop of this solution on a microscope slide and covered with a glass coverslip. The cells were then examined under visible and ultraviolet light and the proportion of fluorescent cells was determined.

2.3.1.6 Calculation of mean relative growth rate

The mean relative growth rate was determined by the following equation:

$$MRGR = \frac{\log_e W_2 - \log_e W_1}{T_2 - T_1}$$

where:

MRGR = Mean Relative Growth Rate
W_1 = initial fresh weight of cells
W_2 = final fresh weight of cells
$T_2 - T_1$ = time that the cells were in culture

(The results were expressed as $d.^{-1} \times 10^2$)

2.3.2 Analysis of Culture Medium

2.3.2.1 Determination of sucrose concentration

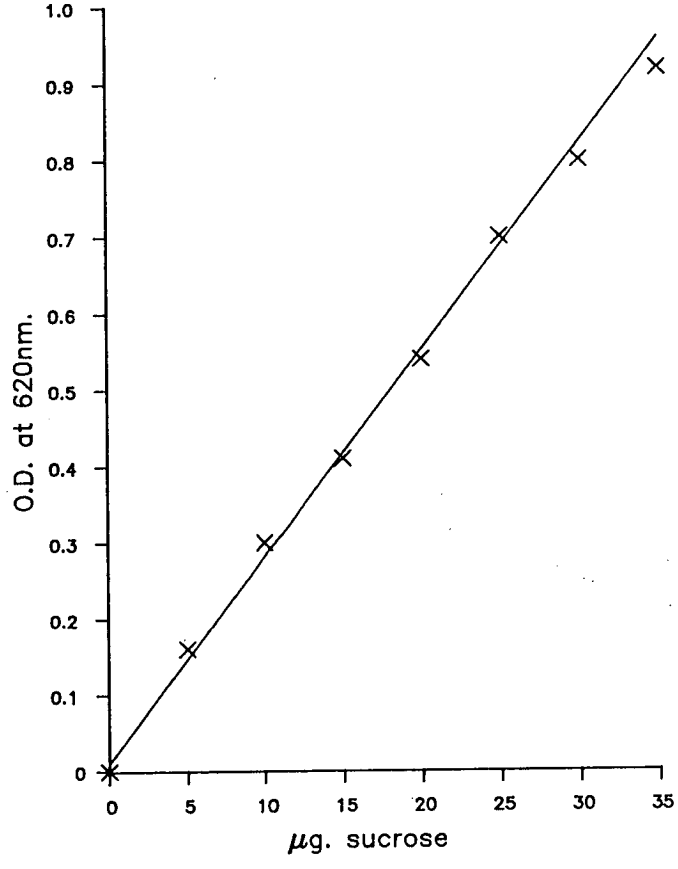
Sucrose levels in the medium were measured using the 'Anthrone method' as described by Ashwell (1957). For each analysis fresh anthrone reagent, 0.2% w/v anthrone (Sigma Chemical Company, Poole, Dorset) in conc. H_2SO_4 (BDH Ltd., Poole, Dorset), was prepared. 1ml. of this reagent was added to 0.5ml. of a suitably diluted sample of medium. These were mixed thoroughly and placed in a boiling water bath for 5min. The resulting green coloured solutions were cooled and the optical density of the samples were measured at 620nm. using a colorimeter. A typical calibration curve obtained using standard sucrose solutions is shown in Fig. 2.3.1.

2.3.2.2 Determination of the osmolality

The osmolality ($osmol.kg.^{-1}$) of the medium was measured using a Clandon-Gonotec 030 osmometer (Clandon Scientific Ltd., Aldershot, Hampshire). This measures the total osmolality of aqueous solutions by making comparative measurements of the freezing points of pure water and of solutions. The machine was calibrated using distilled water (0.0 $osmol.kg.^{-1}$) and a standard solution of known osmolality (0.319 $osmol.kg.^{-1}$). The samples were then measured.

Figure 2.3.1

Calibration curve for sucrose.



2.3.3 Analysis of the Carotenoid Content of Cultures and Plant Tissue

2.3.3.1 Extraction of carotenoids

Carotenoids were extracted from both the plant and tissue culture samples using the method described by Davies (1976). A known fresh weight of tissue was homogenized using a pestle and mortar with 80% acetone (v/v in distilled water). The homogenate was vacuum filtered through a 2.4cm. glass microfibre filter (Whatman International Ltd., Maidstone) and the residue recovered for further extraction. The procedure was repeated until all the pigment had been extracted. An equal volume of diethyl ether (BDH Ltd., Poole, Dorset) was added to the combined acetone extracts in a separating funnel along with sufficient distilled water to produce a bilayer. The mixture was shaken and when the bilayer reformed the lower (aqueous) phase was removed. The aqueous phase was re-extracted with diethyl ether until it ceased to be coloured (*ca.* 3 times). The diethyl ether solutions (containing the carotenoid pigments) were combined and washed with water 3 times to remove any traces of acetone. The water was removed and the resulting diethyl ether extract was dried using anhydrous sodium sulphate (*ca.* 10g./50ml. extract) (BDH Ltd., Poole, Dorset). The drying agent was filtered off, washed with diethyl ether and the combined diethyl ether solution was concentrated (*in vacuo*) to a small volume at room temperature. The sample was then dried under a stream of nitrogen using minimal heating. All samples were stored in dark glass vials, under nitrogen at -20°C.

2.3.3.2 Thin layer chromatography

Reverse phase glass plates (KC_{18}, Whatman International Ltd., Maidstone) were used for all thin layer chromatography (TLC). The solvent system used for both plant tissue and culture extracts was methanol : acetic acid (499:1). Sealed TLC tanks were equilibrated with the solvent system 1-2h. prior to use. During this time, the plates were loaded using a Hamilton glass syringe (capacity 10µl.). Standards of bixin, β-carotene, lutein and zeaxanthin (Roth, West Germany) were used. All of these standards and the samples were dissolved in chloroform. The loaded plates were run for 45min. in the solvent, removed and allowed to dry thoroughly before further analysis.

To identify and quantify the pigments the coloured areas were carefully scraped off the TLC plates. The adsorbent so obtained was placed in Eppendorf tubes and 0.5ml. ethyl acetate (BDH) was added. These were centrifuged in a micro-centifuge

for 2min. The ethyl acetate extract was removed and placed in dark vials. This was repeated until all the colour was removed from the adsorbent. The ethyl acetate was evaporated off under a stream of nitrogen then the samples were sealed and stored under nitrogen at -20°C.

2.3.3.3 Measurement and identification of carotenoids

The samples stored at -20°C. (2.3.3.1 and 2.3.3.2) were removed from the freezer and allowed to reach room temperature. 1ml. of chloroform was then added to each sample. The visible absorption spectra of the samples (in chloroform) were measured using a Pye-Unicam SP8-100 spectrophotometer. From the spectra the relative amount of pigment was determined. In some cases the pigment could also be identified from its absorption maxima. Absorption spectra of standards are shown in Fig. 2.3.2.

2.3.3.4 Determination of the proportion of pigmented cells

Before the proportion of pigmented cells could be determined it was necessary to subject both the plant tissue and cultures to a protoplast isolation procedure in order to separate the cells. Chromic acid (section 2.3.1.3) could not be used as this destroyed the pigment in the cells.

Plant tissue and suspension culture cells (*ca.* 50mg. fresh weight) were incubated in 0.5ml. of medium containing CPW salts with 13% mannitol (Sigma Chemical Co., Poole, Dorset) (CPW13M medium) for 1h. The composition of the medium is given in Table 2.3.1. Prior to this incubation the plant tissue was cut into 1-2mm. transverse sections. After 1h. the CPW13M medium was removed with a Pasteur pipette and 0.5ml. of enzyme solution was added. The enzyme solution contained 2% hemicellulase (Sigma Chemical Co., Poole, Dorset), 2% cellulase ('Ononuka' RS) and 2% macerozyme (R-10, Yakult Honsha Co. Ltd., Japan) dissolved in CPW13M medium at pH 5.8. This was incubated overnight (*ca.* 16h.) at 25°C. The solution was then homogenized gently using a Pasteur pipette and the cells were counted using a haemocytometer (2.3.1.3). Both pigmented and non-pigmented cells were counted and the proportion of pigmented cells was determined.

Figure 2.3.2

Spectra (600-320nm.) of (i) Bixin, (ii) β-carotene, (iii) Zeaxanthin and (iv) Lutein standards.

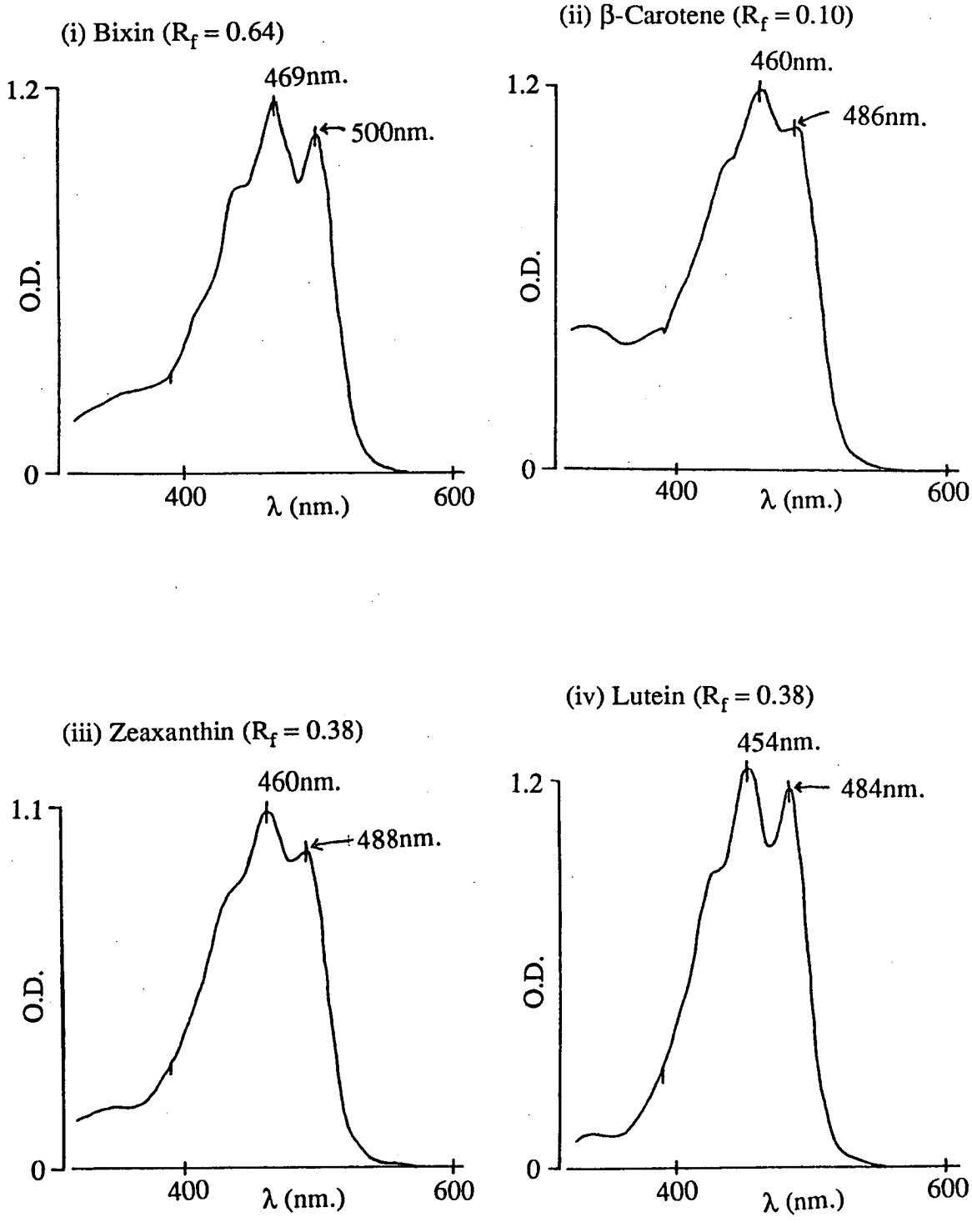


Table 2.3.1

The composition of CPW13M medium (Reinert and Yeoman, 1982).

Constituents	Concentration in Media ($mg.l.^{-1}$)
$CaCl_2.2H_2O$	1480
$CuSO_4.5H_2O$	0.025
KH_2PO_4	27.2
KI	0.16
KNO_3	101
$MgSO_4.7H_2O$	246
mannitol	130,000
pH	5.8

2.3.4 Analysis of Pigment Distribution in the Plant

Hand sections of plant tissue were prepared by cutting very thin sections with a single edged razor blade. Both transverse and longitudinal sections were prepared. Some of these sections were placed on a microscope slide mounted in water with a glass coverslip. However, some pigment tended to smear across the section making it difficult to determine the exact location of the pigment. To overcome this some of the sections were washed in oil and some in ethanol. The ethanol removed all of the pigment whereas the oil only removed some. By comparing these sections the location of the pigment was determined.

2.3.5 Statistical Analyses

In the majority of experiments three replicates were used for each treatment and the mean of these values was calculated. To determine the amount of variation within a treatment the standard error of the mean value was calculated. Also the means of two samples were compared. Observed differences between means were determined using "Student's t-test" as it is suitable for the small samples (n=<30) used in this investigation.

Standard errors were calculated and Student t-tests were performed according to Parker (1979). However, when the data was in the form of percentages, the values were subjected to arcsin transformation and statistical manipulations were carried out on the transformed values (see Sokal and Rohlf, 1969; Snedecor and Cochran, 1971). This was done because percentage values do not conform to the normal distribution which means that statistical methods which assume that sample data are normally distributed about a mean cannot be applied to their analysis. Zero percentage values were given a transformed value of 0.57 degrees which has been found to improve the equality of variance in the angles (see Snedecor and Cochran, 1971). Results were presented as arcsin transformed data ($\arcsin\sqrt{P}$) in all cases, with the corresponding percentage values indicated in the graphs for convenience. Tables found in Rohlf and Sokal (1969) were used for the transformations.

Finally linear regression as described by Parker (1979) was used to fit a regression line to data for the sucrose calibration curves (section 2.3.2.1). The regression equation representing how much y changes with any given change of x can be used to construct a regression line on a scatter diagram and in the simplest case this is assumed to be a straight line. The equation of the straight line is of the form $\mathbf{y = a + bx}$, where **b** defines the gradient and **a** the point at which the regression line crosses the y axis.

Chapter 3

Results

3.1 THE NATURE AND DISTRIBUTION OF CAROTENOID PIGMENTS IN THE PLANT

Orange carotenoid pigments are present in many parts of *Bixa orellana* but seeds of the plant are the commercial source of the carotenoid bixin. The appearance of seeds of *B.orellana* showing the orange/red pigment layer on the outside of the seeds is shown in Fig. 3.1.1. The experiments in this section were conducted to determine the nature and distribution of the plant pigment and to discover whether bixin was present in parts other than the seeds. A study was made of the range of carotenoid pigments present, identifying the location of these pigments and estimating the proportion of pigmented cells in different parts of the plant. Also, the pigments present in the plant tissues were examined using TLC and visible spectra analysis.

3.1.1 Determination of the Location of Carotenoid Pigments in the Plant

Carotenoid pigments have been observed in the leaf, stem, petiole and root of *B.orellana* but the exact location of the pigments was not known. Therefore, the aim of this experiment was to determine the location of the pigment(s).

Hand sections of root, leaf, stem and petiole from three month old *B.orellana* plants were prepared as described in 2.3.4. Root sections were also prepared from 3-4d. old seedlings since this organ was routinely used to initiate callus. When the sections were placed on a microscope slide mounted in water some orange pigment smeared across the section making it difficult to determine its location. The pigment could be removed either by washing the sections in ethanol or olive oil. Ethanol removed all of the pigment whereas oil only removed some. The sections were examined microscopically and by comparing the sections in water with those washed in oil or ethanol the location of the pigment was determined. The location of the pigment in the four different organs examined is shown in Fig. 3.1.2. Also the appearance of pigmented cells in the cortex of a longitudinal stem section is shown in Fig. 3.1.3.

Pigmented cells were observed in all of the plant organs examined (Fig. 3.1.2(a), (b) and (c)). In the root, stem and petiole the pigment was located in the cortex and the phloem whereas in the leaf the pigment was present in the spongy mesophyll and phloem. In all of the sections examined the pigmented cells were randomly distributed within particular tissues. In the cortex and spongy mesophyll the pigmented cells (40.8μm.) were *ca.* 3x larger than those in the phloem (12μm.). When the sections were cut the pigment appeared to move to the cut surface which

suggests that there may be some form of channel in which the pigment can move. The number of pigmented cells appeared to vary in the different tissues with the greatest number in the three month old roots. However, in this experiment the actual numbers of pigmented cells were not determined. In the next experiment the proportion of pigmented cells was determined in each of the four organs.

Figure 3.1.1

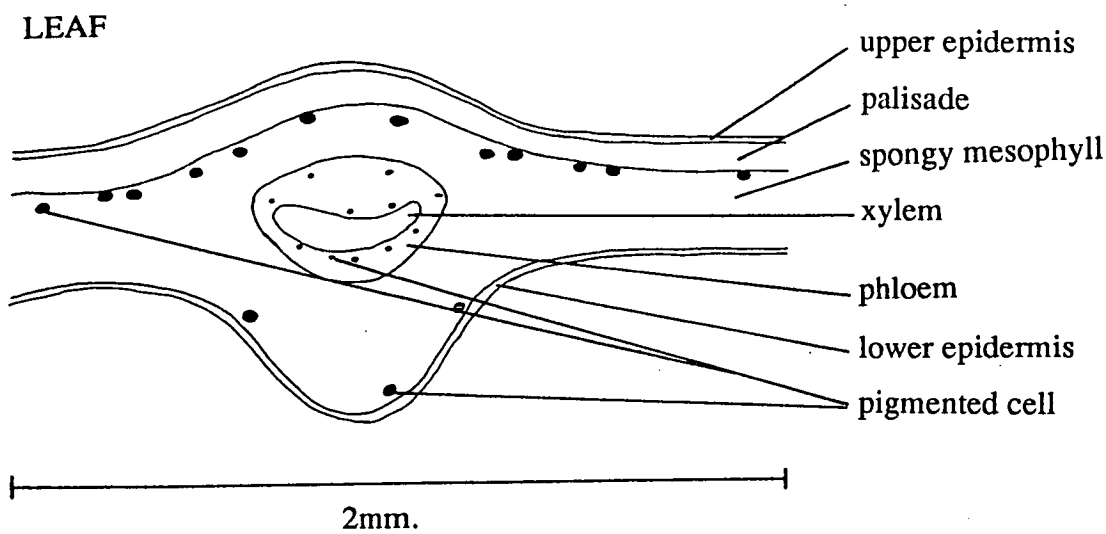
The appearance of seeds of *B.orellana* showing the orange/red layer of pigment on the outside of the seeds.

5mm

Figure 3.1.2

Diagrams of transverse sections of leaf, petiole (a); stem, root (3 months old) (b); and root from 3-4d. old seedlings (c) of *B.orellana* showing the location of orange/red pigmented cells. Pigmented cells are shown as black areas.

Figure 3.1.2(a)



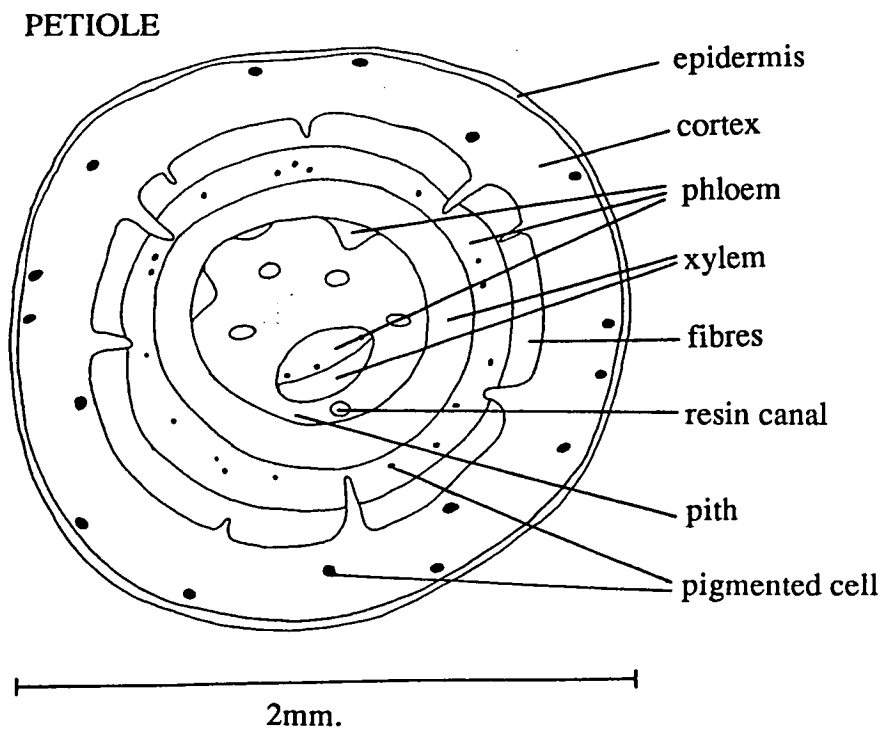
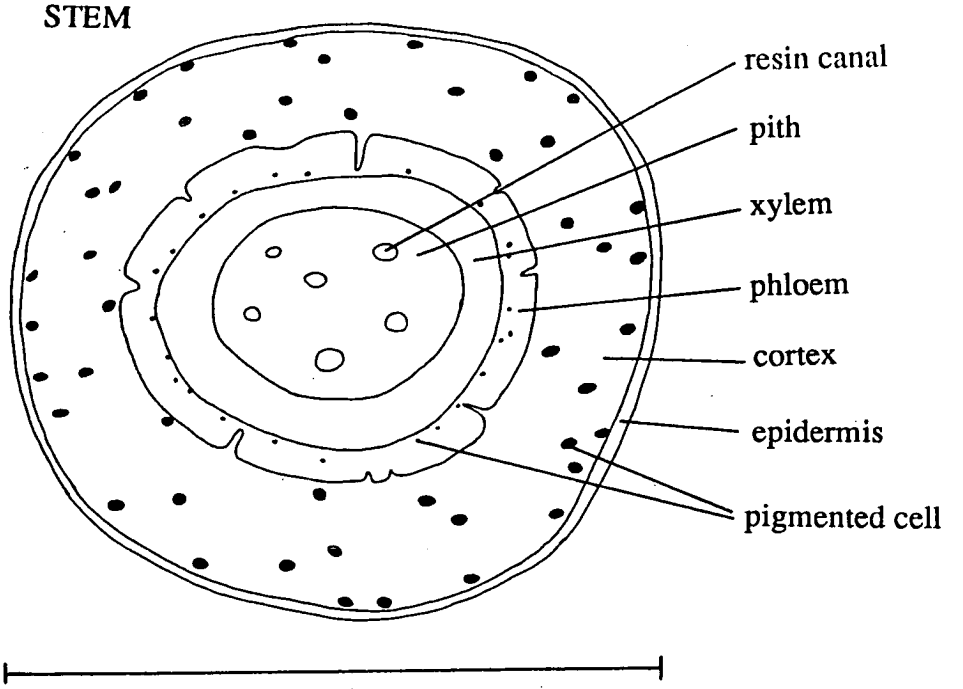


Figure 3.1.2(b)

STEM



ROOT (3 months old)

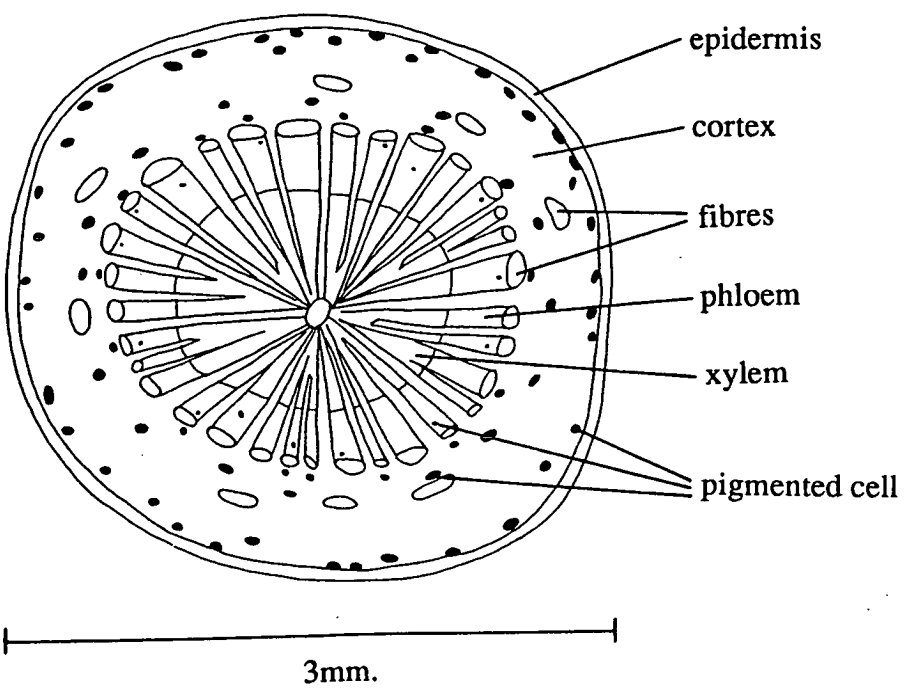


Figure 3.1.2(c)

ROOT (3-4d. old seedling)

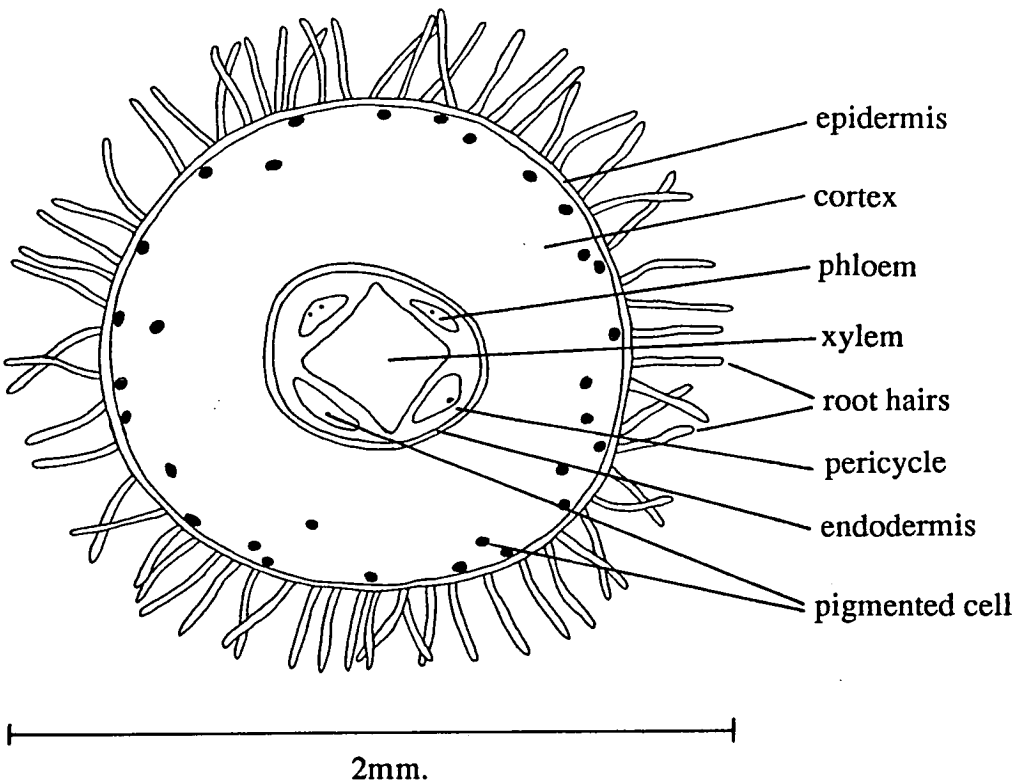
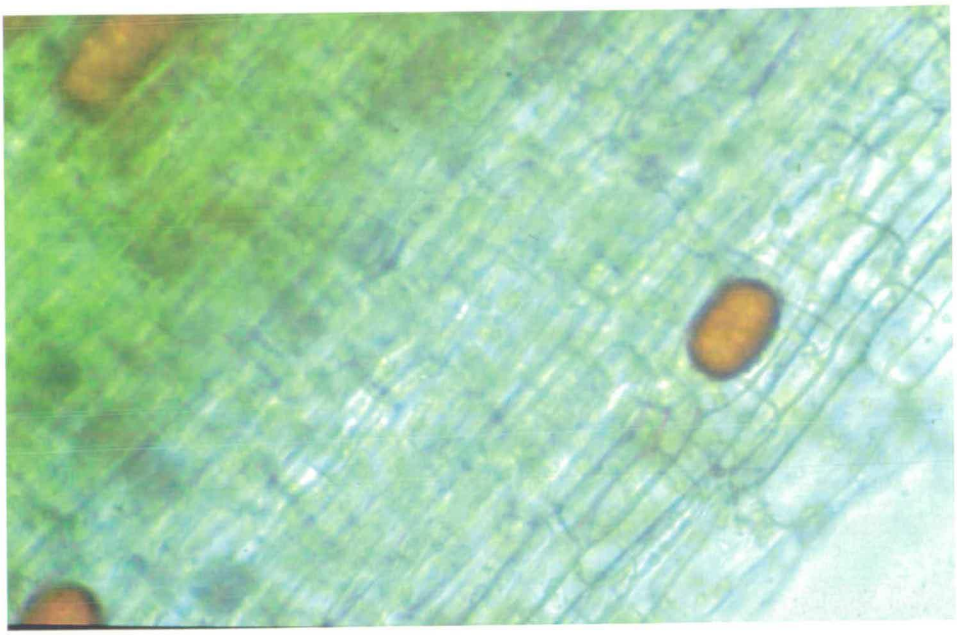


Figure 3.1.3

The appearance of pigmented cells in the cortex of a longitudinal stem section of *B.orellana.*



3.1.2 Estimation of the Proportion of Pigmented Cells in Plant Organs

In the previous experiment (3.1.1) the location of carotenoid pigments in the tissues of various plant organs of *B.orellana* was determined. It was also observed that the number of pigmented cells appeared to vary in the different tissues. The aim of this experiment was to estimate the proportion of pigmented cells in the root, stem, leaf and petiole.

Roots, stems, leaves and petioles from three month old plants and roots from 3-4d. old seedlings were used in this experiment. To estimate the proportion of pigmented cells *ca.* 50mg. of plant tissue was sliced into thin transverse sections (1-2mm.). These were then incubated in CPW13M medium (see Table 2.3.1) for 1h. followed by overnight incubation in an enzyme solution (2% hemicellulase, 2% macerozyme and 2% cellulase) as described in 2.3.3.4. At the end of the incubation period the suspended cells were carefully separated using a Pasteur pipette and were then counted using a haemocytometer (see 2.3.1.3). The appearance of a pigmented cell obtained by this method is shown in Fig. 3.1.4. The proportion of pigmented cells was determined and the results are presented in Table 3.1.1.

The proportion of pigmented cells was very low and no significant differences were found between the five samples (Table 3.1.1). Since the proportion of pigmented cells in roots from 3-4d. old seedlings was not significantly different to the proportion in three month old roots it would appear that the number of pigmented cells in root tissue did not change with time. Having identified the location of the carotenoid pigment and estimated the proportion of pigmented cells in these plant organs the next experiment was designed to determine the nature of this pigment(s) using TLC and visible spectra analysis.

Figure 3.1.4

The appearance of a pigmented cell obtained from stem tissue of *B.orellana*.

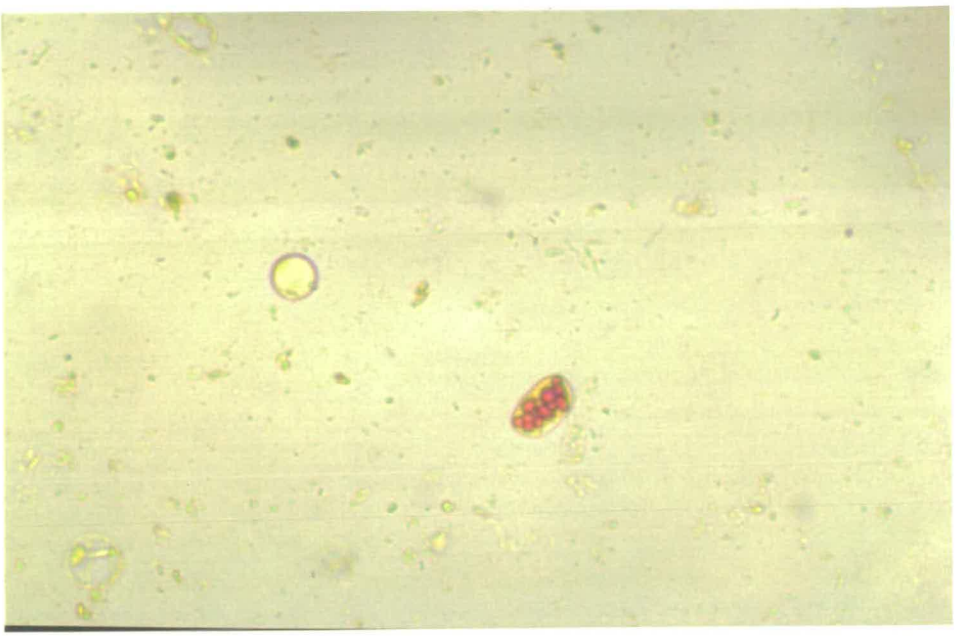


Table 3.1.1

The proportion of pigmented cells in root, leaf, petiole and stem of *B.orellana*.

Plant Organ	Proportion of Pigmented Cells (%)
Stem (3 months)	3.8
Petiole (3 months)	2.5
Leaf (3 months)	3.0
Root (3 months)	4.0
Root (3-4d. seedling)	2.2

3.1.3 Analysis of the Carotenoid Pigments in Plant Tissues of *B.orellana*

In the last two experiments (3.1.1 and 3.1.2) carotenoid pigments were located in various parts of *B.orellana* plants and the proportion of pigmented cells in the different organs was determined. The aim of this experiment was to extract these carotenoids and analyse them using TLC and visible spectra analysis.

Carotenoids were extracted from *ca.* 2g. of root, leaf, stem and petiole as described in 2.3.3.1. The leaf, stem and petiole material was from three month old plants whereas the roots from 3-4d. old seedlings were used as these were readily available for extraction. The pigment was also extracted from seeds of *B.orellana* by stirring the seeds in chloroform until all of the pigment was removed (*ca.* 3h.). The visible absorption spectra of the samples obtained was measured (in chloroform) prior to TLC analysis (see 2.3.3.3). 200µl. of the samples in chloroform were then loaded onto a reverse phase TLC plate (KC_{18}). A standard solution containing bixin, β-carotene, lutein and zeaxanthin was also loaded onto the plate. The loaded plates were run for 45min. in a solvent system containing methanol : acetic acid (499:1) as described in 2.3.3.2. After 45min. the plates were removed and allowed to dry. The orange/yellow coloured spots were then scraped off the TLC plate and the pigment extracted using ethyl acetate (see 2.3.3.2). The ethyl acetate was evaporated off

under a stream of nitrogen and the visible absorption spectra of the samples (in chloroform) were measured as described in 2.3.3.3. Table 3.1.2 shows the O.D. for one of the absorption maxima (λ_{max}) values obtained for each pigment. The appearance of a TLC plate and absorption spectra of the plant extracts and the coloured spots obtained in TLC are shown in Figs. 3.1.5 and 3.1.6 respectively.

Fig. 3.1.5 shows that after TLC ten orange/yellow spots were obtained from the leaf, stem and petiole but only nine spots were obtained from the root. There was no spot 8, which from its R_f value (0.38) corresponds to the xanthophyll standards (lutein and zeaxanthin), in the root. Spots 1, 2, 3, 4, 5, 6, 7, 9 and 10 were similar in the root, stem, leaf and petiole. The seed extract contained only one spot which was shown to be bixin from its R_f value (0.64) and visible spectrum. The spectrum obtained was identical to that for standard bixin (see Fig. 2.3.2). Bixin was also present in the root, stem, leaf and petiole (spot 4) but it was not pure as can be seen from the visible spectrum (Fig. 3.1.6). The absorption maxima at 500nm. and 464-467nm. for spot 4 were probably due to bixin. However, spot 4 also had absorption maxima at 419-421nm., 398-401nm. and 375-380nm. Similar maxima were found in spots 1, 2, 3 and 5. These spots were very close together on the TLC plate so it may be that the same compound is present in each of them. The compound(s) may be a breakdown product(s) of bixin. M^{c}Keown (1963, 1965) isolated a yellow pigment from seeds (λ_{max} 428, 404 and 385nm. in chloroform), this was a degradation product formed during the extraction of bixin at high temperatures. Attempts to separate the unknown yellow compound(s) from bixin were not successful. From the spectra (Fig. 3.1.6), spots 1, 2, 3 and 5 appeared to contain more than one compound. Spot 8 which was obtained from the leaf, stem and petiole had a spectrum and R_f value (0.38) similar to lutein (see Fig. 2.3.2 for spectrum of lutein). From the R_f value (0.10) β-carotene appeared to be present in the root, stem, leaf and petiole (spot 10) but from the spectrum (Fig. 3.1.6) it was seen that it was also not pure. A spectrum of a β-carotene standard is shown in Fig. 2.3.2.

Table 3.1.2 shows the O.D. at an absorption maximum for each of the coloured spots. Two λ_{max} values are listed for spot 4, the value at 398-401nm. for comparison with spots 1, 2, 3 and 5 and the value at 500-501nm. which probably represents bixin. The largest value for each spot except for spot 4 (500-501nm.) was for the leaf extract. The highest value for spot 4 (500-501nm.) was for the seed extract. With the exception of spot 6 the petiole spots had higher O.D. values than the stem spots. For spots 1, 2, 6 and 10 the smallest O.D. values were for the root extract. However, the root O.D. values for spots 3, 4 and 5 were higher than both the stem and petiole

values. For the photosynthetic organs the leaf had the highest O.D. values for all of the spots followed by the petiole then the stem. This is what would be expected since the leaf is the primary photosynthetic organ of the plant.

In this experiment the nature of the plant carotenoid pigments has been elucidated and some of the pigments have been identified. Bixin was present in all parts of the plant examined but the highest amount was from the seeds. The xanthophyll lutein was present in the leaf, stem and petiole whereas β-carotene was detected in the stem, leaf, petiole and root of the plant. The pigments in spots 1, 2, 3, 5, 6, 7 and 9 were not identified.

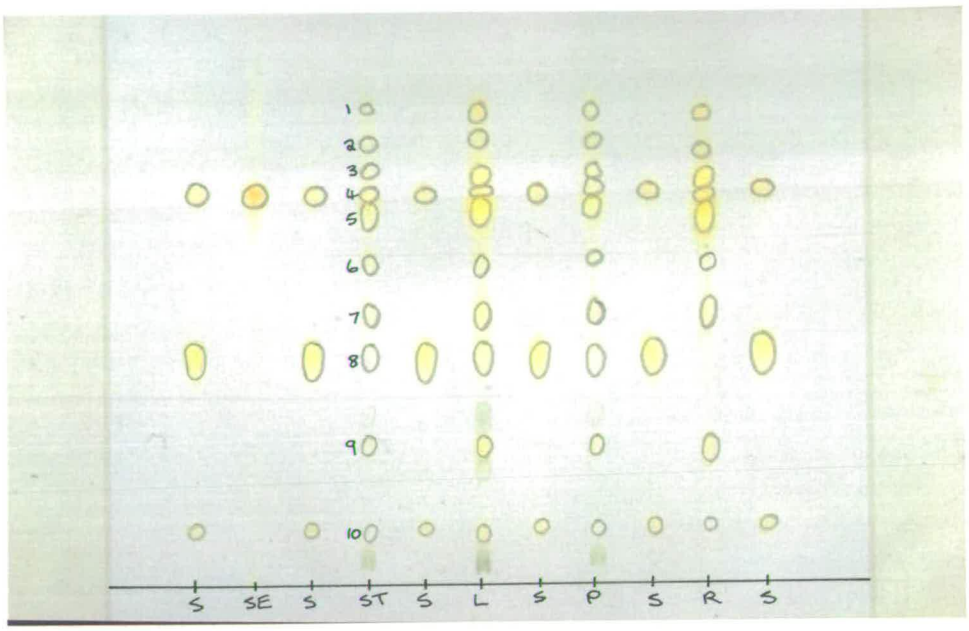
Table 3.1.2

O.D. values at a λ_{max} for each of the orange/yellow carotenoid pigments obtained from the root, stem, leaf, petiole and seeds of *B.orellana.*

Spot Number	λ_{max} nm.	O.D. at λ_{max} per g. fresh weight				
		Root	Leaf	Petiole	Stem	Seed
1	402-404	1.1	30.9	2.8	2.6	-
2	401-402	2.7	28.1	5.2	3.9	-
3	401-402	11.0	25.0	4.5	3.4	-
4	398-401	14.5	52.0	4.2	4.0	-
	500-501	5.1	7.8	2.1	2.3	6595
5	399-400	26.0	230.4	12.4	12.0	-
6	423-425	1.1	20.6	2.2	3.3	-
7	460-463	2.4	26.7	3.6	2.3	-
8	453-454	-	26.0	2.7	1.9	-
9	458-462	2.3	25.8	3.5	1.6	-
10	452-458	0.04	4.7	0.6	0.3	-

Figure 3.1.5

The appearance of a TLC plate obtained from TLC analysis of plant extracts of *B.orellana.*



S = standards (4 = bixin, 8 = zeaxanthin and lutein, 10 = β-carotene)

SE = seed extract

ST = stem extract

L = leaf extract

P = petiole extract

R = root extract

Figure 3.1.6

Spectra obtained for spots 1, 2, 3, 4 (a); spots 5, 6, 7, 8 (b); and spots 9 and 10 (c) from TLC analysis of plant extracts of *B.orellana*.

Figure 3.1.6(a)

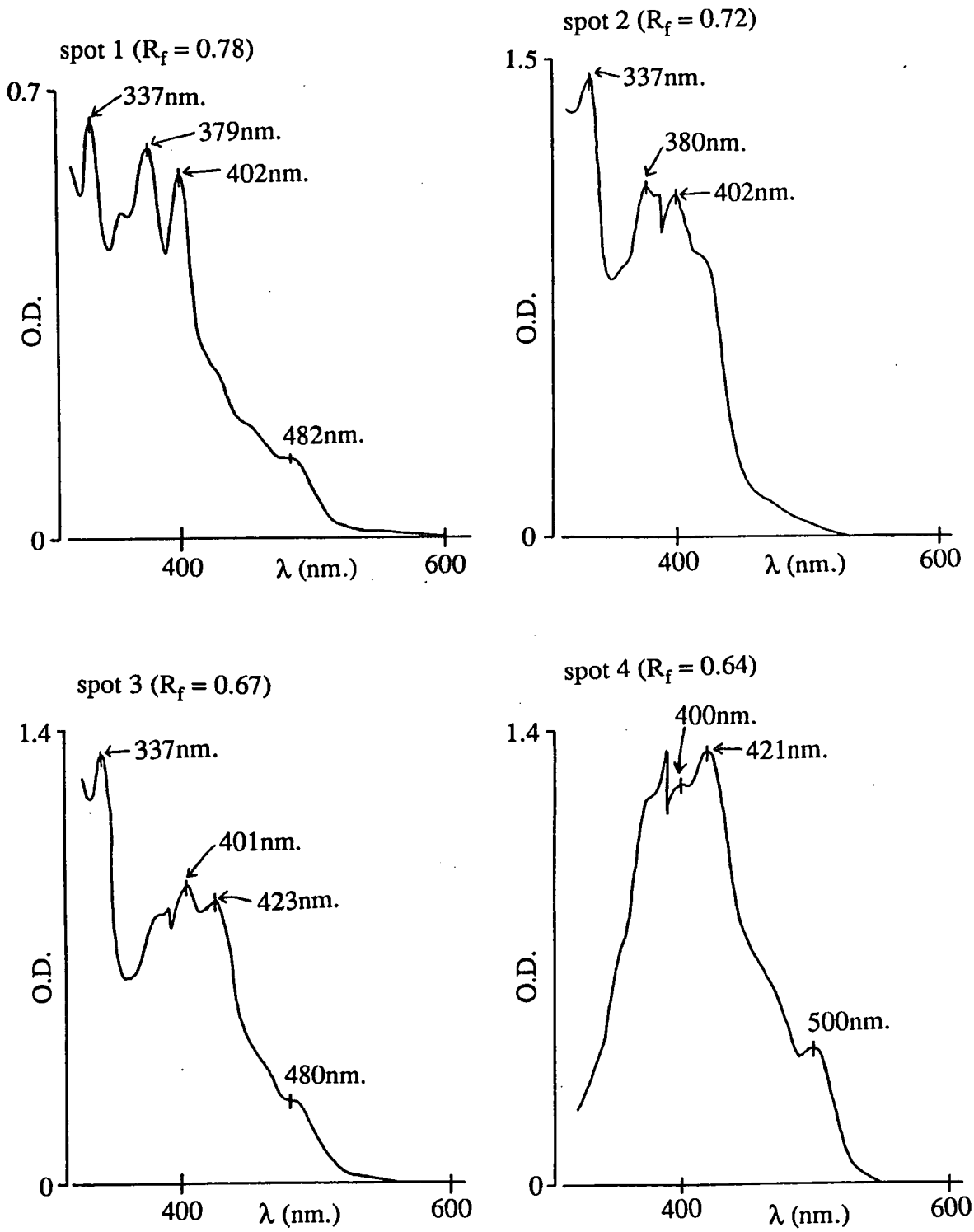


Figure 3.1.6(b)

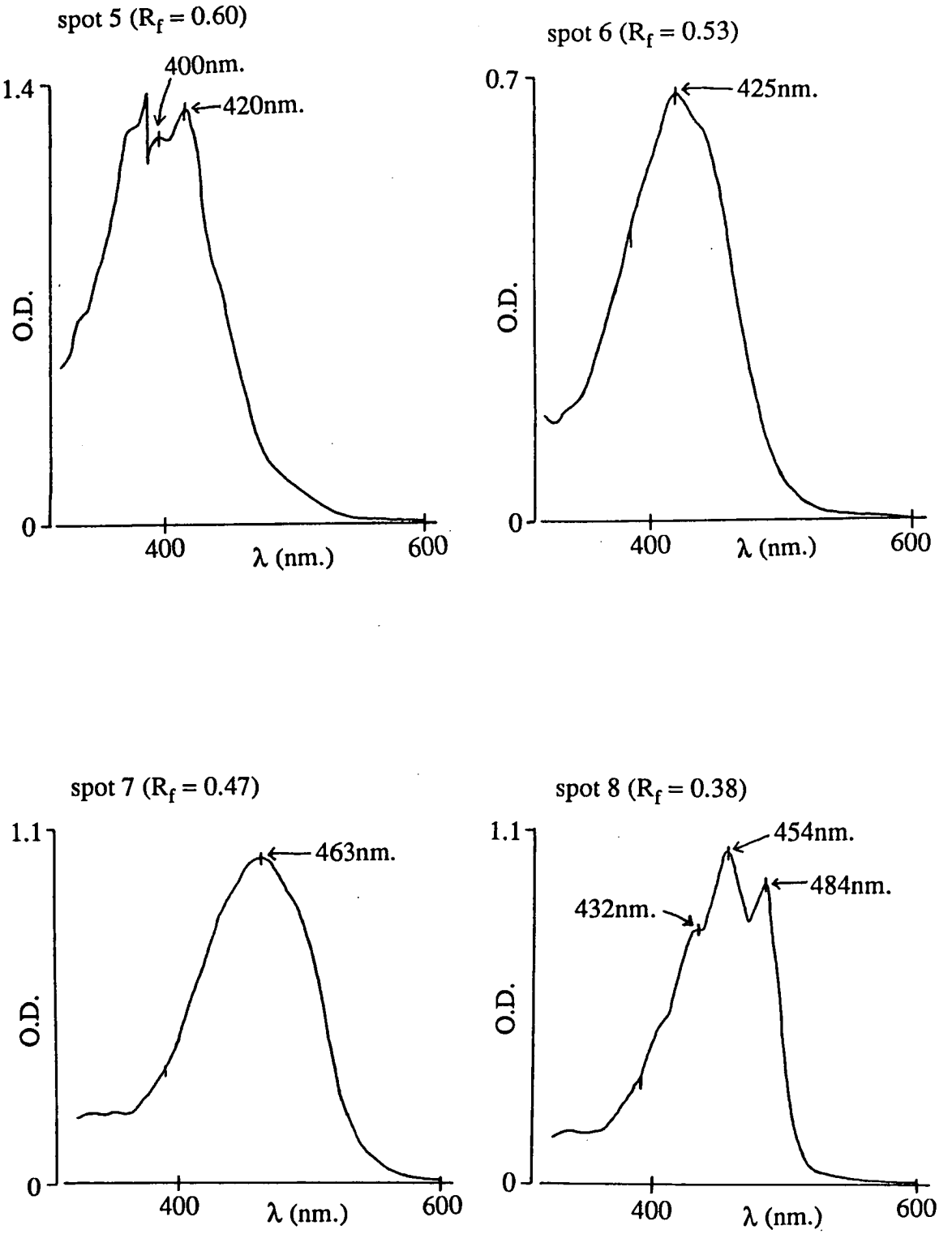
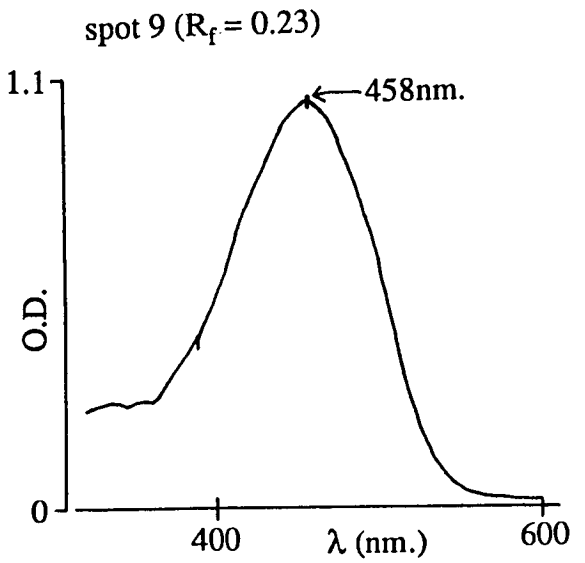
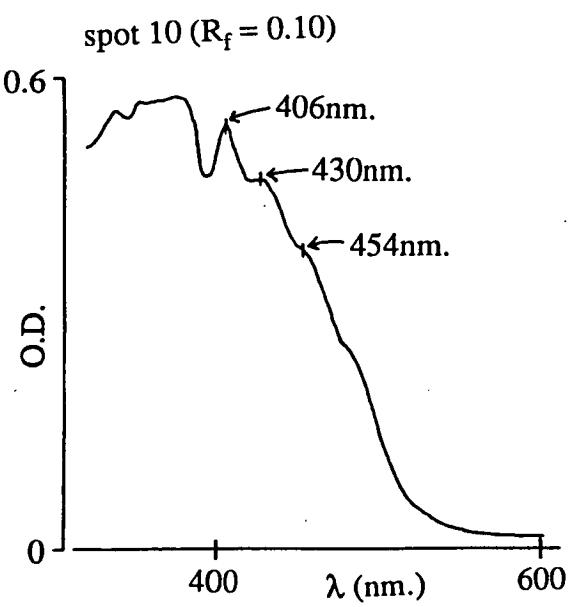

spot 5 (R_f = 0.60)
400nm.
420nm.
1.4
O.D.
0
400
λ (nm.)
600
spot 6 (R_f = 0.53)
0.7
425nm.
O.D.
0
400
λ (nm.)
600
spot 7 (R_f = 0.47)
1.1
463nm.
O.D.
0
400
λ (nm.)
600
spot 8 (R_f = 0.38)
1.1
454nm.
484nm.
432nm.
O.D.
0
400
λ (nm.)
600

Figure 3.1.6(c)


spot 9 (R_f = 0.23)
1.1
458nm.
O.D.
0
400
λ (nm.)
600


spot 10 (R_f = 0.10)
0.6
406nm.
430nm.
454nm.
O.D.
0
400
λ (nm.)
600

Summary of the Results in Section 3.1

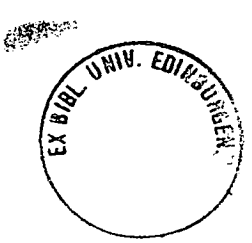
The following notable points have arisen from the work reported in this section.

(1) Pigmented cells are present in the cortex and the phloem of stems, petioles and roots of *B.orellana* and in the spongy mesophyll and phloem of leaves.

(2) The proportion of pigmented cells in the plant tissues examined was very low and not significantly different from each other.

(3) Bixin was present in the leaf, petiole, stem, root and on the seeds of *B.orellana* but the highest level was on the seeds.

Since the root, stem, leaf and petiole of *B.orellana* were all found to contain several carotenoids including bixin, in the next section these tissues will be used to initiate cultures and the production of carotenoid pigments by these cultures will be investigated.



3.2 ESTABLISHMENT OF CULTURES OF *B.ORELLANA*

In the previous section it was shown that a variety of carotenoids, including bixin, were present in the roots, stems, petioles and leaves of *B.orellana.* In order to investigate the production of carotenoids in tissue cultures it was first of all necessary to initiate and establish callus cultures and to optimize the culture conditions paying particular attention to the level of growth regulators, the best nutrient medium and source of explant. Subsequently conditions were optimized for the initiation and establishment of suspension cultures from callus.

3.2.1 Establishment of Callus Cultures

Prior to the work reported in this study callus cultures of *B.orellana* had already been initiated from stem tissue of 8-12 week old plants and were being subcultured every four weeks. The medium used is listed in Table 3.2.1. There were problems with this medium:

1. Root regeneration occurred on a regular basis

2. The callus was very brown in appearance

3. Coconut milk was a constituent of the culture medium and difficulties were encountered in the reproducibility between different batches of medium

The first approach was to optimize and balance the amount of plant growth regulators in the medium to minimize organogenesis and maximize callus growth (Skoog and Miller, 1957).

Table 3.2.1

The composition of the original medium used for the initiation and growth of *B.orellana* callus.

Constituents	Concentration In Medium
Murashige and Skoog (1962) Medium	$4.71g.l.^{-1}$
NAA	$0.5mg.l.^{-1}$
Coconut milk	$20ml.l.^{-1}$
Sucrose	$30g.l.^{-1}$
Agar	$10g.l.^{-1}$
pH	5.8

3.2.1.1 A preliminary experiment to show the effect of added cytokinin on the initiation and growth of callus cultures

In the original cultures of *B.orellana* taken over at the outset of this study root regeneration occurred frequently indicating that the cytokinin level in the medium may have been too low to sustain good callus growth (Skoog and Miller, 1957). Indeed the only cytokinin present in the medium was presumably provided by the coconut milk (Table 3.2.1). The aim of this experiment was to prevent regeneration and improve the growth of the callus by the addition of various cytokinins to the culture medium.

Explants (transverse sections, 4mm. long) were cut from sterile stem tissue (2.2.2.3) obtained from 8-12 week old plants. An explant size of 4mm. was previously found to be the minimum amount of tissue required to initiate callus. Single explants were placed on 15ml. of agar medium in sterile 5cm. Petri dishes and callus was initiated as described in 2.2.3.3. Five different media were used:

a. original medium (OM), see Table 3.2.1

b. OM + 2-IP, - coconut milk

c. OM + KIN, - coconut milk

d. OM + 6-BAP, - coconut milk

e. OM + ZEA, - coconut milk

Coconut milk is a natural product and the variability between batches makes it difficult to achieve reproducible results. The concentration of cytokinin used in each case was 5 x 10^{-7}M (*ca.* 0.1mg.l.$^{-1}$) as kinetin is typically added at this concentration for the induction of callus (Dodds and Roberts, 1985). After four weeks the callus formed was removed, weighed and subcultured onto fresh agar medium. Three replicate samples were prepared for each medium. After a further four weeks the callus was weighed and the mean relative growth rates were determined as described in 2.3.1.6. The results are shown in Table 3.2.2.

Callus was initiated on all of the media and, in general, when cytokinin was present in the medium white, friable callus was obtained and no root regeneration was observed in the cultures. Table 3.2.2 shows that during the first subculture the callus grew best when 2-IP or 6-BAP was used. When 2-IP or 6-BAP were present the mean relative growth rate was significantly higher than on the original medium (2-IP at P=0.01 and 6-BAP at P=0.001). The mean relative growth rate when ZEA or KIN was present was not significantly different from the original medium. However, when cytokinin was added to the culture medium the callus was white instead of brown.

Although callus growth was improved when 2-IP or 6-BAP was added to the culture medium, it was still low. A doubling of fresh weight in 28d. would represent a mean relative growth rate of 2.48 x 10^{-2} d.$^{-1}$ after 28d. therefore, none of the callus cultures doubled in fresh weight during the culture period. In the next experiment the concentration of the cytokinins 2-IP and 6-BAP, shown to be the most effective in promoting callus growth, were varied in an attempt to increase callus growth further.

Table 3.2.2

Mean relative growth rates for callus cultures on five different media during the first subculture after callus initiation. Each value is the mean of three replicates ± s.e.

Medium	Mean Relative Growth Rate ($d.^{-1} \times 10^2$)
OM	0.19 ± 0.05
OM + 2-IP, - coconut milk	1.37 ± 0.19
OM + KIN, - coconut milk	0.25 ± 0.12
OM + 6-BAP, - coconut milk	1.19 ± 0.06
OM + ZEA, - coconut milk	0.36 ± 0.12

3.2.1.2 Optimization of cytokinin concentration for initiation and growth of callus cultures

In the previous experiment (3.2.1.1) it was found that when cytokinin was added to the culture medium white, friable callus was obtained, root regeneration was prevented and the growth of callus was increased significantly with 2-IP and 6-BAP. However, the growth of callus, although improved, was still poor. The aim of this experiment was an attempt to further increase the growth of callus cultures by altering the concentration of 2-IP and 6-BAP.

Sterile stem explants (4mm.) were prepared and callus was initiated as described in the previous experiment (3.2.1.1). Six different media were used to initiate and grow callus cultures. All of the media contained the same constituents as the original medium (Table 3.2.1) except that coconut milk was absent and cytokinin was added as follows:

A. 1 x 10^{-6}M 2-IP

B. 5 x 10^{-7}M 2-IP *

C. 2.5 x 10^{-7}M 2-IP

D. 1 x 10^{-6}M 6-BAP

E. 5 x 10^{-7}M 6-BAP *

F. 2.5 x 10^{-7}M 6-BAP

* These media were used in the previous experiment.

The explants were weighed prior to callus initiation. After 28d. the tissue (explant and callus) was weighed and the callus removed, weighed and placed on fresh agar medium. Each sample contained *ca.* 200 - 300mg. of callus tissue. After 21d. the colour of the cultures changed from white to brown indicating that they had exhausted the nutrients in the medium and were dying. Therefore, after 21d. the callus was weighed and the mean relative growth rates obtained during initiation and the first subculture were calculated. The results are shown in Table 3.2.3.

The amount of callus tissue produced was very variable and this results in large standard errors (s.e.s). The results show (Table 3.2.3) that there were no significant differences in the mean relative growth rates during callus initiation for any of the treatments. However, less callus tissue was produced when 6-BAP was present in the medium which meant that for the first subculture only one or two samples were available and the s.e.s could not be calculated. The highest mean relative growth rate in the first subculture was for 1 x 10^{-6}M 2-IP. This was significantly higher than the value for 5 x 10^{-7}M 2-IP (at P=0.1), but it was not known whether it was significantly different from the samples on 6-BAP. The largest values for the growth rate were obtained when the medium contained 2-IP: 5 x 10^{-7}M during callus initiation and 1 x 10^{-6}M during the first subculture.

In general, the mean relative growth rates were higher in this experiment than in the previous one. In this experiment the callus in the first subculture was harvested after 21d. because it was turning brown. Previously (3.2.1.1), the cultures were harvested after 28d. by which time they may have been dying since in this experiment they were turning brown after 21d. This could account for the lower growth rates in the previous experiment (3.2.1.1). Therefore, in future experiments

the callus was subcultured after 14d. because 21d. was too long. Also, 2-IP was used in future experiments since the highest mean relative growth rates were obtained and more callus was produced with this cytokinin. The optimum concentration of 2-IP was not determined because the results were variable. In the next experiment the optimum concentrations of 2-IP and NAA in the culture medium required to obtain maximal initiation and growth of callus cultures were determined.

Table 3.2.3

Mean relative growth rates for the initiation and first subculture of callus cultures on six different media.

Medium	**Mean Relative Growth Rate ($d.^{-1} \times 10^2$)**	
	Initiation	**First Subculture**
A : 1 x 10^{-6}M 2-IP	7.40 ± 0.98	7.25 ± 0.70
B : 5 x 10^{-7}M 2-IP	8.80 ± 1.23	4.18 ± 0.95
C : 2.5 x 10^{-7}M 2-IP	7.77 ± 1.04	3.50 ± 1.73
D : 1 x 10^{-6}M 6-BAP	7.51 ± 1.18	6.18 *
E : 5 x 10^{-7}M 6-BAP	7.25 ± 0.71	5.09 *
F : 2.5 x 10^{-7}M 6-BAP	6.95 ± 0.60	6.29 *

* Only one or two samples were obtained so the s.e.s could not be calculated.

3.2.1.3 Optimization of both the cytokinin and auxin concentrations for the initiation and growth of callus cultures

In the previous two experiments (3.2.1.1 and 3.2.1.2) it was shown that the addition of 2-IP to the culture medium improved callus initiation and growth. Indeed some of the largest increases in fresh weight occurred with 2-IP. However, the optimum concentration of 2-IP was not determined as the results were variable. Therefore, the aim of this experiment was to vary both the cytokinin (2-IP) and auxin (NAA) level in the medium to optimize the conditions for initiation and growth of callus cultures.

Explants were cut from sterile stem tissue and callus was initiated as described in the previous experiments (3.2.1.1 and 3.2.1.2). In this experiment nine different media were tested. These contained the same constituents as the original medium (Table 3.2.1) except that coconut milk was absent, auxin (NAA) and cytokinin (2-IP) were added at different concentrations. The growth regulators present in the media were as follows:

1. 2.5 x 10^{-7} M 2-IP, 5.4 x 10^{-6}M NAA

2. 2.5 x 10^{-7}M 2-IP, 2.7 x 10^{-6}M NAA *

3. 2.5 x 10^{-7}M 2-IP, 1.35 x 10^{-6}M NAA

4. 5 x 10^{-7}M 2-IP, 5.4 x 10^{-6}M NAA

5. 5 x 10^{-7}M 2-IP, 2.7 x 10^{-6}M NAA *

6. 5 x 10^{-7}M 2-IP, 1.35 x 10^{-6}M NAA

7. 1 x 10^{-6}M 2-IP, 5.4 x 10^{-6}M NAA

8. 1 x 10^{-6}M 2-IP, 2.7 x 10^{-6}M NAA *

9. 1 x 10^{-6}M 2-IP, 1.35 x 10^{-6}M NAA

* These media were used in the previous experiment.

The explants were weighed prior to callus initiation. After 28d. the tissue was weighed then the callus was removed, weighed and subcultured onto fresh medium (*ca.* 70 - 80mg. of callus per sample). The callus was left for 14d. then it was

weighed and subcultured. A time interval of 14d. was employed because previously the callus had turned brown after 21d. (3.2.1.2). The procedure was repeated so that the results for three subcultures were obtained. This allowed the cultures to be studied over a longer time period than in previous experiments. The mean relative growth rates were calculated as described in 2.3.1.6 and the results are shown in Table 3.2.4.

Callus was initiated on all of the media and the mean relative growth rates during initiation were not significantly different between the treatments. In general, the results show that the mean relative growth rates decreased with successive subcultures. However, the rate of decrease varied depending on the medium. The mean relative growth rate for the first subculture was significantly less than the value during initiation for media 1, 2, 3, 4, 6, 8 and 9 (at P=0.1 for medium 3; P=0.05 for media 2, 6, 8 and 9; P=0.01 for media 1 and 4). Media 5 and 7 did not decline significantly until the third subculture (at P=0.05 for medium 7; P=0.001 for medium 5). Overall, the results were very variable but it would appear that media 5 and 7 gave the best growth rates over several subcultures. Both these media gave white, friable callus. When compared to the results of the previous experiment it can be seen that decreasing the culture period from 21d. to 14d. resulted in improved growth of the cultures.

Since media 5 and 7 were equally good at initiating and growing callus these were both used in further experiments to optimize the conditions for callus initiation and growth.

Table 3.2.4

Mean relative growth rates for the initiation and subculture (x3) of callus cultures on nine different media.

Medium	Mean Relative Growth Rate ($d.^{-1} \times 10^2$)			
	Initiation	**First Subculture**	**Second Subculture**	**Third Subculture**
1 : 2.5 x 10^{-7}M 2-IP	6.88	3.27	3.93	3.19
5.4 x 10^{-6}M NAA	± 0.69	± 0.66	± 0.71	± 0.44
2 : 2.5 x 10^{-7}M 2-IP	7.63	4.63	4.33	3.17
2.7 x 10^{-6}M NAA	± 0.71	± 0.86	± 0.40	± 0.95
3 : 2.5 x 10^{-7}M 2-IP	7.13	4.39	4.73	2.41
1.35 x 10^{-6}M NAA	± 0.35	± 0.82	± 0.73	± 0.61
4 : 5 x 10^{-7}M 2-IP	8.15	4.63	4.92	4.34
5.4 x 10^{-6}M NAA	± 0.85	± 0.63	± 0.74	± 0.33
5 : 5 x 10^{-7}M 2-IP	7.18	6.27	7.13	4.45
2.7 x 10^{-6}M NAA	± 0.63	± 0.39	± 0.33	± 0.53
6 : 5 x 10^{-7}M 2-IP	7.70	5.20	4.14	1.89
1.35 x 10^{-6}M NAA	± 0.74	± 0.93	± 0.33	± 0.46
7 : 1 x 10^{-6}M 2-IP	7.72	6.82	7.11	4.73
5.4 x 10^{-6}M NAA	± 0.41	± 0.74	± 0.57	± 0.94
8 : 1 x 10^{-6}M 2-IP	8.48	5.31	4.79	4.11
2.7 x 10^{-6}M NAA	± 0.79	± 0.79	± 1.33	± 0.72
9 : 1 x 10^{-6}M 2-IP	8.08	6.37	4.53	4.07
1.35 x 10^{-6}M NAA	± 0.38	± 0.68	± 0.74	± 0.69

3.2.1.4 Effect of the basic nutrient medium on the growth of callus cultures

Previously it was found that two media (5 and 7) were equally good at initiating and supporting the growth of callus cultures (3.2.1.3). The aim of this experiment was to test the growth of callus on different nutrient media to determine which was best.

Callus which had been initiated from stem tissue and grown on medium 5 (see 3.2.1.3) was used in this experiment. Three media were used: MS medium, SH medium and White's medium (see 2.2.1.2 and 2.2.1.3). The growth regulators in each medium were the same as in medium 5: 5 x 10^{-7}M 2-IP and 2.7 x 10^{-6}M NAA. A known weight of callus (*ca.* 50mg.) was weighed and subcultured onto 15ml. fresh agar medium in 5cm. Petri dishes. The Petri dishes were sealed with parafilm and maintained under standard culture conditions (see 2.2.3.3). After 14d. the callus was weighed and subcultured again onto fresh agar medium. This procedure was repeated for four subcultures. The mean relative growth rates were determined from the fresh weight measurements as described in 2.3.1.6. The results are presented in Table 3.2.5.

The results show that SH medium was not significantly different from the MS medium for the first three subcultures. However, for the fourth subculture the mean relative growth rate for SH medium was less than MS medium. The growth rate for White's medium was less than both MS and SH media throughout the culture period. Overall, MS medium appears to be the best nutrient medium for callus growth. Therefore, in the next experiment MS medium was used to test the ability of different plant tissues to initiate callus.

Table 3.2.5

Mean relative growth rates for four subcultures of callus cultures on three different nutrient media.

Medium	Mean Relative Growth Rate ($d.^{-1} \times 10^2$)			
	First Subculture	**Second Subculture**	**Third Subculture**	**Fourth Subculture**
MS	3.77	4.59	5.16	6.91
	± 0.70	± 0.84	± 1.16	± 1.16
SH	2.53	3.72	5.05	3.18
	± 0.48	± 0.71	± 0.89	± 0.41
White's	2.17	2.79	1.11	1.47
	± 0.35	± 0.53	± 0.22	± 0.56

3.2.1.5 Effect of explant origin on the initiation and growth of callus cultures

Previously (see 3.2.1.3) two media (5 and 7) were found to give callus which was white, friable and had a high growth rate over several subcultures. Also MS medium was found to be the best nutrient medium for callus growth (3.2.1.4). In these experiments stem tissue was always used to initiate the callus (see 3.2.1.1, 3.2.1.2 and 3.2.1.3). The aim of this experiment was to test other explants for their ability to initiate callus. The growth of the callus over several subcultures was also investigated.

Stem and leaf tissue from 8-12 week old plants was sterilized as described in 2.2.2.3(b) then stem explants (transverse sections, 4mm. long) and 1cm. leaf discs were cut and callus was initiated as described in 2.2.3.3. Seeds of *B.orellana* were sterilized using the method described in 2.2.2.3(a) and they were germinated. Root

explants (transverse sections, 4mm. long) were then cut from 3-4d. seedlings and callus was initiated as described in 2.2.3.3. Two media were used: MS medium with 5 x 10^{-7}M 2-IP and 2.7 x 10^{-6}M NAA (medium 5), MS medium with 1 x 10^{-6}M 2-IP and 5.4 x 10^{-6}M NAA (medium 7). Ten replicate samples were prepared for each treatment and the explants were weighed prior to callus initiation. Callus was initiated under standard culture conditions (see 2.2.3.2 and 2.2.3.3). After 28d. the tissue (explant and callus) was weighed (2.3.1.1) and the callus was removed, weighed and transferred to fresh agar medium. After a further 14d. the callus was weighed aseptically and transferred once again to fresh medium. This procedure was repeated for a further two subcultures. The mean relative growth rates during initiation and the three subcultures were determined as described in 2.3.1.6 and the results are presented in Table 3.2.6.

Friable, white callus was obtained from root and stem tissue on both media (Table 3.2.6(a) and Table 3.2.6(b)) whereas the leaf callus tissue was grey and hard. During initiation the root tissue gave the highest mean relative growth rates on both media. The lowest value was obtained for leaf tissue on medium 5. The mean relative growth rate for leaf callus increased during the first subculture then decreased on both media. The values for root callus decreased during the three subcultures on both media. The growth rate for stem callus on medium 5 in the first subculture was not significantly different from the initiation value, but it then decreased after the first subculture. The stem callus on medium 7 decreased over the three subcultures. In this experiment it was observed that pigmented callus was produced from root and stem tissue. However, the pigment could not be analysed because the level was low and the amount of callus tissue available for extraction was limited. The appearance of the pigmented callus is shown in Fig. 3.2.1.

It would appear from these results that root tissue was best for callus initiation. However, both root and stem tissue produced friable callus which was pigmented. On medium 7 the stem callus grew better than the root callus in the first two subcultures. However, on medium 5 both root and stem callus grew equally well and the growth rates were not significantly different during the three subcultures. Leaf callus gave the highest mean relative growth rates during the first subculture but the callus was not friable and no pigment was seen. Therefore, in future experiments root or stem tissue was used for callus initiation and growth. Once again both media were equally good for callus initiation and growth. In the next experiment both media were used to initiate suspension cultures.

Table 3.2.6

Mean relative growth rates of callus initiated on medium 5 (5 x 10^{-7}M 2-IP and 2.7 x 10^{-6}M NAA) (a) and medium 7 (1 x 10^{-6}M 2-IP and 5.4 x 10^{-6}M NAA) (b) from the root, stem and leaf of *B.orellana* during initiation and three subcultures.

Table 3.2.6(a) (Medium 5)

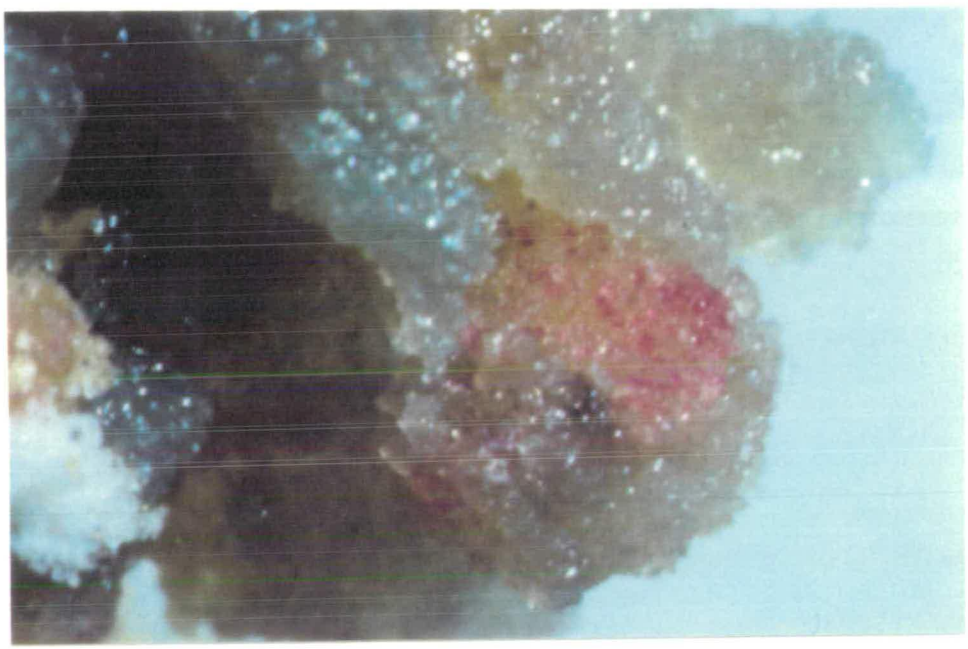
Plant Tissue	**Mean Relative Growth Rate ($d.^{-1}$ x 10^2)**			
	Initiation	**First Subculture**	**Second Subculture**	**Third Subculture**
Root	12.88	6.94	4.16	1.19
	± 1.88	± 1.13	± 0.36	± 0.24
Stem	8.12	7.41	3.50	1.40
	± 0.49	± 0.71	± 0.36	± 0.14
Leaf	6.64	8.35	2.69	1.16
	± 0.96	± 0.78	± 0.15	± 0.18

Table 3.2.6(b) (Medium 7)

Plant Tissue	**Mean Relative Growth Rate ($d.^{-1}$ x 10^2)**			
	Initiation	**First Subculture**	**Second Subculture**	**Third Subculture**
Root	11.66	3.67	2.63	1.01
	± 0.78	± 0.60	± 0.55	± 0.40
Stem	9.52	7.78	4.34	1.27
	± 0.24	± 0.76	± 0.30	± 0.21
Leaf	8.51	10.59	4.17	1.24
	± 1.04	± 0.42	± 0.39	± 0.11

Figure 3.2.1

The appearance of pigmented callus cultures of *B.orellana*.



3.2.2 The Establishment of Suspension Cultures

In the previous experiments (3.2.1) callus cultures of *B.orellana* were established and two media were shown to be equally effective for the initiation and growth of callus. In the following experiments suspension cultures were established and the batch growth of these cultures was investigated.

3.2.2.1 Initiation of suspension cultures of *B.orellana*

In experiment 3.2.1.3 two media were found to initiate and support growth of callus equally well (media 5 and 7). The aim of this experiment was to determine the optimum inoculum density required for initiating suspension cultures using both these media.

Two media were used in this experiment: MS with 5 x 10^{-7}M 2-IP and 2.7 x 10^{-6}M NAA (medium 5), MS with 1 x 10^{-6}M 2-IP and 5.4 x 10^{-6}M NAA (medium 7). Due to lack of callus tissue 100ml. conical flasks were used in this experiment so as to maintain a specified biomass / to volume of liquid medium. A range of callus inocula as detailed in Table 3.2.7 were added to 20ml. of the same medium without agar in 100ml. conical flasks. The flasks were then placed on an orbital shaker under standard culture conditions (2.2.3.2). After 7d. the cultures were filtered through a sterile 800μm. nylon sieve to remove any lumps (2.2.3.4) and the cells and medium which passed through the sieve were left for a further 7d. on the orbital shaker in 100ml. flasks. The packed cell volume (PCV) and viability of the cultures were measured at time 0d., 7d. and 14d. as described in 2.3.1.4 and 2.3.1.5. The results are presented in Table 3.2.7.

The results for medium 5 (Table 3.2.7(a)) show that the viability of the cultures decreased sharply over the 14d. for all the inocula. As a result of this the PCV for medium 5 increased by only a very small amount between d.0 and d.14. For medium 7 (Table 3.2.7(b)) the viability for all the inocula also decreased in the first 7d. but the decrease was much smaller than for medium 5. Between d.7 and d.14 the viability increased for the 1.0g. inoculum on medium 7 but it decreased for the remaining three levels of inoculum. The PCV increased over the 14d. for all of the inocula on medium 7. The increases in PCV for medium 7 were much larger than those for medium 5, with the largest increase for the 1.0g. inoculum (1.8 fold) on medium 7.

Medium 7 gave better results overall than medium 5. Since the viability of the

cultures decreased very quickly and suspension cultures were difficult to initiate on medium 5, this medium was not used subsequently for suspension cultures. However, medium 7 was used routinely for initiating and maintaining both callus and suspension cultures of *B.orellana* and was known as the standard culture medium or MS medium (see 2.2.1.2). Based on these results an inoculum of 1.0g. was used for the initiation of suspension cultures.

Having established a suspension culture of *B.orellana* it was now essential to maximize growth of these cultures and this is described in the next experiment.

Table 3.2.7(a)

The viability and PCV of suspension cultures of *B.orellana* at three different time points during initiation from four different amounts of inoculum in medium 5 (MS medium with 5 x 10^{-7}M 2-IP and 2.7 x 10^{-6}M NAA).

Inoculum (g.)	Viability (%)			PCV (%)		
	0d.	7d.	14d.	0d.	7d.	14d.
0.5	89.3	45.0	24.3	4.5	3.5	4.0
1.0	89.3	50.8	31.6	8.0	9.0	10.3
1.5	89.3	51.3	40.2	11.0	10.8	12.5
2.0	89.3	44.9	25.7	15.0	13.5	12.5

Table 3.2.7(b)

The viability and PCV of suspension cultures of *B.orellana* at three different time points during initiation from four different amounts of inoculum in medium 7 (MS medium with 1 x 10^{-6}M 2-IP and 5.4 x 10^{-6}M NAA).

Inoculum (g.)	Viability (%)			PCV (%)		
	0d.	7d.	14d.	0d.	7d.	14d.
0.5	90.0	58.5	54.0	4.5	4.8	7.3
1.0	90.0	73.9	75.0	8.0	8.8	14.2
1.5	90.0	62.4	53.5	11.0	10.8	14.8
2.0	90.0	66.7	53.4	15.0	15.8	16.3

3.2.2.2 Determination of the optimum inoculum density for the subculture of suspension cultures

In the previous experiment (3.2.2.1) medium 7 was found to be the best medium for initiating suspension cultures and the optimum inoculum density was 1.0g. of callus. The aim of this experiment was to determine the inoculum density which gave maximal growth during the subculture of suspension cultures of *B.orellana.*

More tissue was available in this experiment than in 3.2.2.1 so both 100ml. and 250ml. conical flasks were used. Suspension cultures were initiated as described in the previous experiment using standard culture medium (medium 7) (see 2.2.1.2) and 1.0g. inoculum. After 14d. under standard culture conditions (2.2.3.2) the cultures were subcultured into fresh liquid medium. The cultures were combined in a 500ml. sterile conical flask and this was filtered through a 64µm. sieve. The filtered cells were placed in 9cm. sterile Petri dishes then a range of inocula of these filtered cells were placed in 100ml. and 250ml. flasks. Five replicate samples were prepared for the 100ml. flasks and three replicates for the 250ml. flasks. There were fewer replicates for the 250ml. flasks due to lack of tissue. The flasks were placed on a rotary shaker and maintained under standard culture conditions (see 2.2.3.2). The

PCV was measured initially then after a 14d. culture period (see 2.3.1.4). The results are presented in Table 3.2.8.

It would appear from the results presented that there was an optimum size for the inoculum. Above and below this optimum value the increase in PCV was less. Larger increases in PCV were obtained for 250ml. flasks (Table 3.2.8(b)) compared to the equivalent inocula in 100ml. flasks (Table 3.2.8(a)). The optimum for both 250ml. and 100ml. conical flasks was 1.0g. of filtered cells. In the next experiment an inoculum of 1.0g. was used to investigate the batch growth of *B.orellana* suspension cultures.

Table 3.2.8(a)

The effect of inoculum size on the growth of suspension cultures in 100ml. flasks.

Inoculum (g.)	**Initial PCV (%)**	**Final PCV (%)**	**Increase**
0.5	4.5	10.0	2.2 fold
0.75	6.0	17.5	2.9 fold
1.0	8.0	46.0	5.8 fold
1.25	9.5	47.5	5.0 fold
1.5	11.0	54.0	4.9 fold
2.0	15.0	72.0	4.8 fold
3.0	21.5	76.0	3.5 fold
4.0	29.5	78.0	2.6 fold

Table 3.2.8(b)

The effect of inoculum size on the growth of suspension cultures in 250ml. flasks.

Inoculum (g.)	Initial PCV (%)	Final PCV (%)	Increase
0.5	1.8	12.0	6.7 fold
1.0	3.2	38.3	12.0 fold
1.5	4.4	44.7	10.2 fold
2.0	6.0	47.7	8.0 fold
2.5	7.4	46.7	6.3 fold
3.0	8.6	55.7	6.5 fold
4.0	11.8	51.3	4.3 fold
5.0	14.4	53.3	3.7 fold
6.0	17.4	54.3	3.1 fold
7.5	20.0	48.7	2.4 fold
10.0	25.0	47.7	1.9 fold

3.2.2.3 Investigation of the growth of *B.orellana* suspension cultures

Suspension cultures of *B.orellana* have been established and the optimum inoculum density for initiation and growth of these cultures was determined (3.2.2.1 and 3.2.2.2). The aim of this experiment was to measure several parameters of the suspension cultures during batch growth. The chosen parameters were fresh weight, dry weight, viability, PCV, cell number and pH of the medium.

On day 0, the contents of ten 14d. stock suspension cultures previously grown in MS medium (2.2.1.2) in 100ml. flasks were combined aseptically in a 500ml. sterile conical flask. This was mixed thoroughly then filtered (64μm. sieve) and placed in sterile 9cm. Petri dishes. 1g. of filtered cells was added aseptically to 20ml. of sterile

MS medium in 100ml. conical flasks. The flasks were then incubated on a rotary shaker under the conditions described in 2.2.3.2. At regular intervals three flasks were removed at random for analysis. The viability of the cultures was determined as described in 2.3.1.5. The PCV was measured as described in 2.3.1.4 then the cells were filtered (64μm. sieve) and the fresh weight was determined (see 2.3.1.1). Once the fresh weight was measured a small sample of the filtered cells (*ca.* 50mg.) was removed to estimate the cell number (2.3.1.3). The remaining sample was used to determine the dry weight of the culture (see 2.3.1.2). Finally, the pH of the culture medium in each flask was measured using a pH meter. The results are presented in Figs. 3.2.2, 3.2.3, 3.2.4 and 3.2.5.

The results for fresh weight (Fig. 3.2.2(a)) show that there was a lag phase of *ca.* 6d. when no increase in fresh weight occurred. Between d.6 and d.20 there was a 9.8 fold increase in fresh weight which then remained constant between d.20 and d.30. Therefore, the stationary phase was reached by d.20. There was a 1.1 fold decrease in the fresh weight between d.30 and d.40.

During the first 8d. there was no significant change in the dry weight of the cultures (Fig. 3.2.2(b)). After this lag phase the dry weight increased *ca.* 3.5 fold between d.8 and d.16 and then remained constant between d.16 and d.30. Between d.30 and d.40 there was a 1.1 fold decrease in the dry weight.

The data in Fig. 3.2.3(a) show that there was no lag phase for the PCV which increased 11.6 fold between d.0 and d.20. Subsequently there was a 1.1 fold decrease between d.20 and d.22 followed by a 1.1 fold rise between d.22 and d.25. Between d.25 and d.30 the PCV remained constant and then decreased 1.1 fold between d.30 and d.40.

The viability of the cultures (Fig. 3.2.3(b)) decreased 1.1 fold over the first two days and then increased 1.1 fold between d.2 and d.4. After d.4 the viability remained constant until d.14, decreased 1.1 fold between d.14 and d.16 and increased 1.1 fold between d.16 and d.18. The viability remained constant between d.18 and d.25 and decreased 1.8 fold between d.25 and d.40.

Changes in the cell number per flask over the culture period are shown in Fig. 3.2.4(a). These show there was a lag phase of *ca.* 4d. followed by an 11.3 fold rise in cell number between d.4 and d.16. After d.16 the cell number remained constant. The decline between d.22 and d.25 was not significant. Therefore, it would appear from these results that cell division was taking place actively between d.4 and d.16.

The cell number per g. fresh weight (Fig. 3.2.4(b)) was also constant for the first four days and then increased 1.6 fold between d.4 and d.6 before remaining constant between d.6 and d.8. There was a 1.2 fold drop in cell number per g. fresh weight between d.8 and d.10 after which it remained constant again until d.16. A 1.6 fold decline in cell number per g. fresh weight occurred between d.16 and d.20 then it was constant for two days before decreasing 1.3 fold between d.22 and d.25. The number of cells per g. fresh weight was again constant between d.25 and d.30, before it increased between d.30 and d.40 (1.2 fold). These results show that the cells became smaller after an initial lag phase of four days. However, as the culture period progressed the number of cells per g. fresh weight of tissue decreased so the cells became larger. At the end of the culture period the cell number per g. fresh weight increased indicating that the cells became smaller once again.

The pH of the medium was variable during the culture period (Fig. 3.2.5). Between d.0 and d.22 the values ranged between 4.5 and 5.4 with an average value of 4.9. There was a 1.3 fold rise in pH between d.22 and d.40 and on d.30 and d.40 the pH of the medium was above 6.0.

From these results it would appear that between d.0 and d.4 there was a lag phase when the cells were adapting to their new environment and there was no growth or cell division. Between d.4 and d.6 cell division was active but there was no increase in tissue mass. After d.6 both cell division and increase in tissue mass occurred until d.16 when the cells stopped dividing but continued to grow. By d.20 the cells had reached the stationary phase when there was no growth in tissue mass or rise in cell number probably due to lack of essential nutrients in the medium.

Figure 3.2.2

Changes in the fresh weight (a) and dry weight (b) of suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

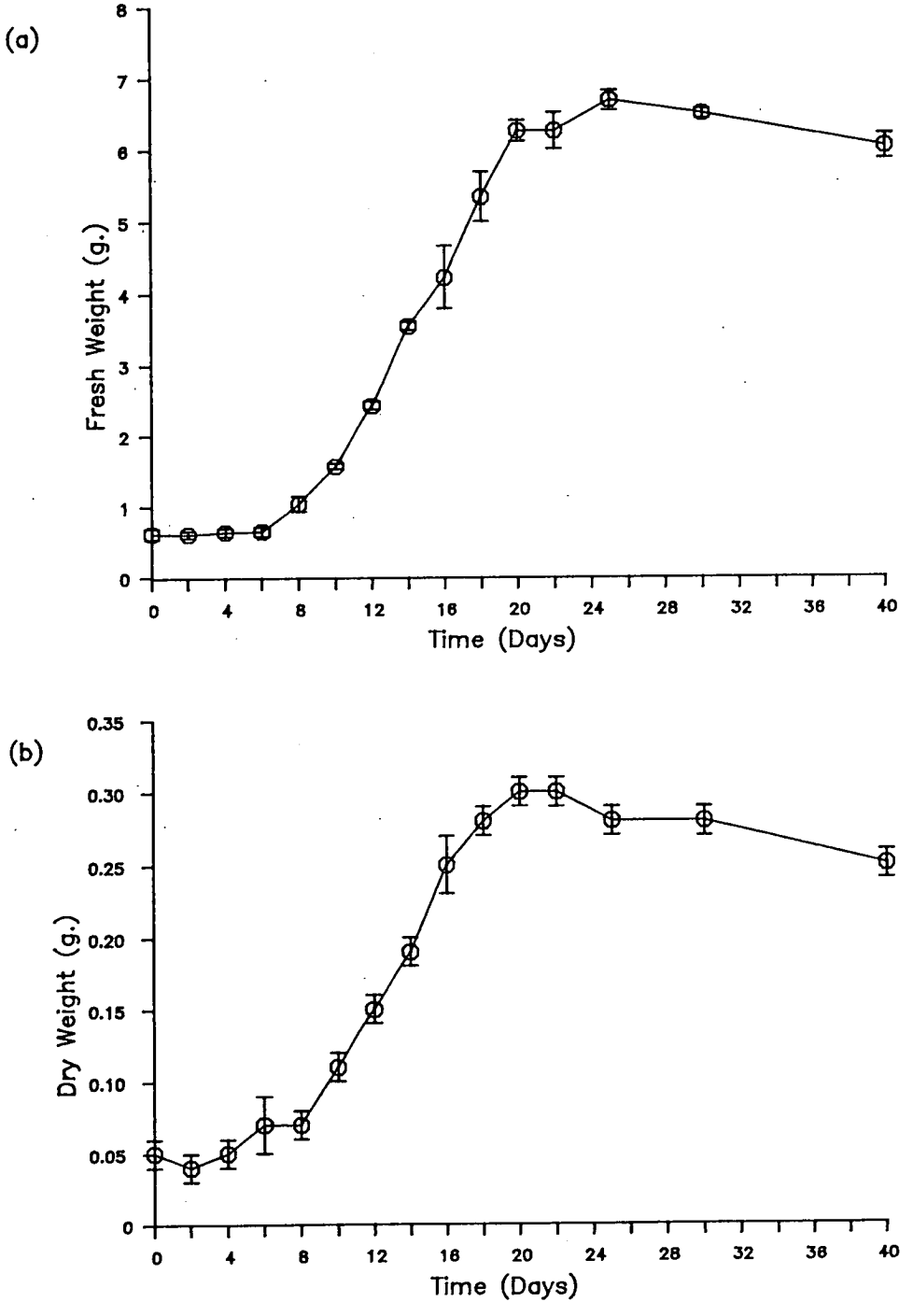


Figure 3.2.3

Changes in the PCV (a) and the viability (b) of suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

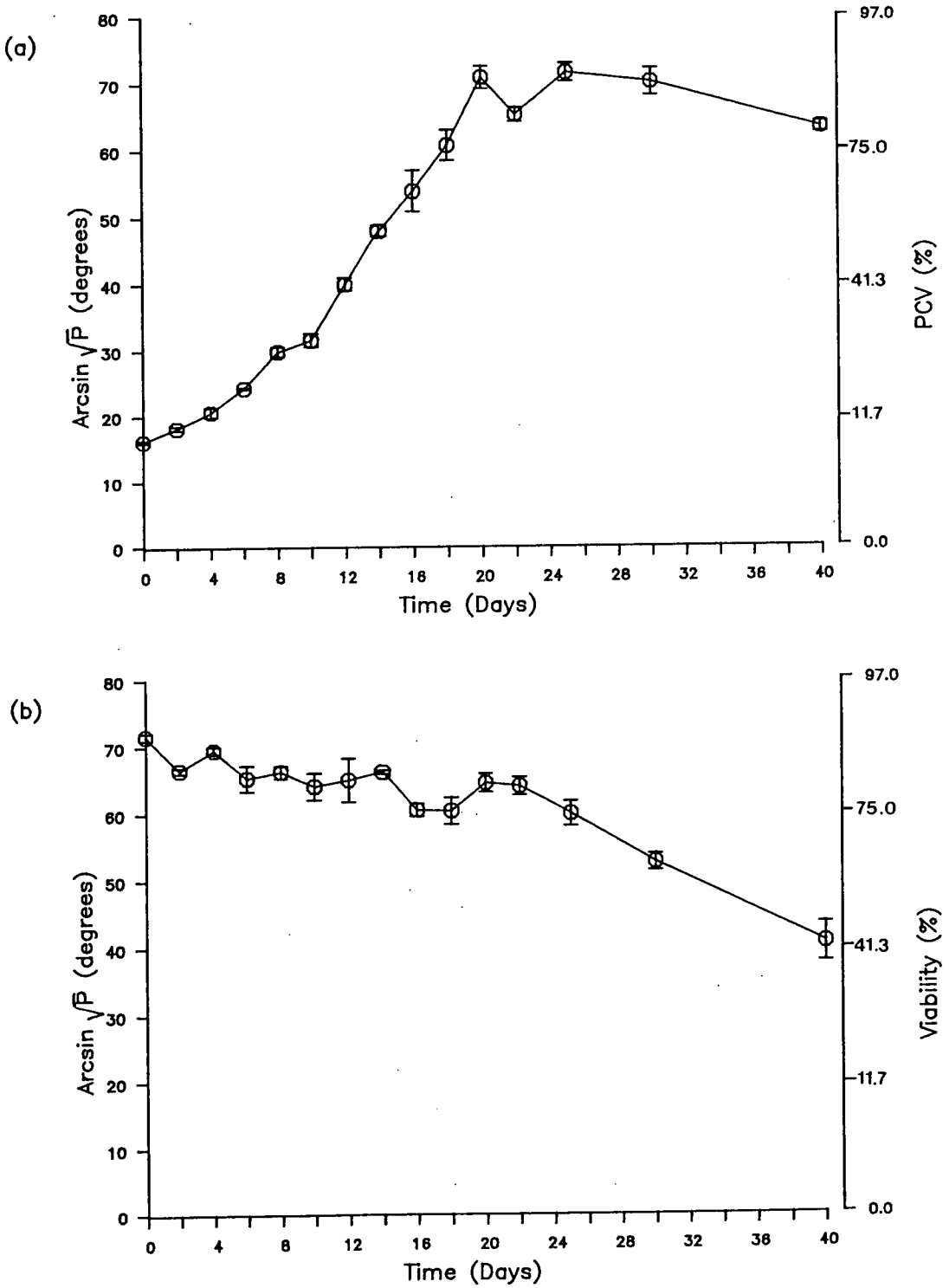


Figure 3.2.4

Changes in cell number per flask (a) and per g.fresh weight (b) in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

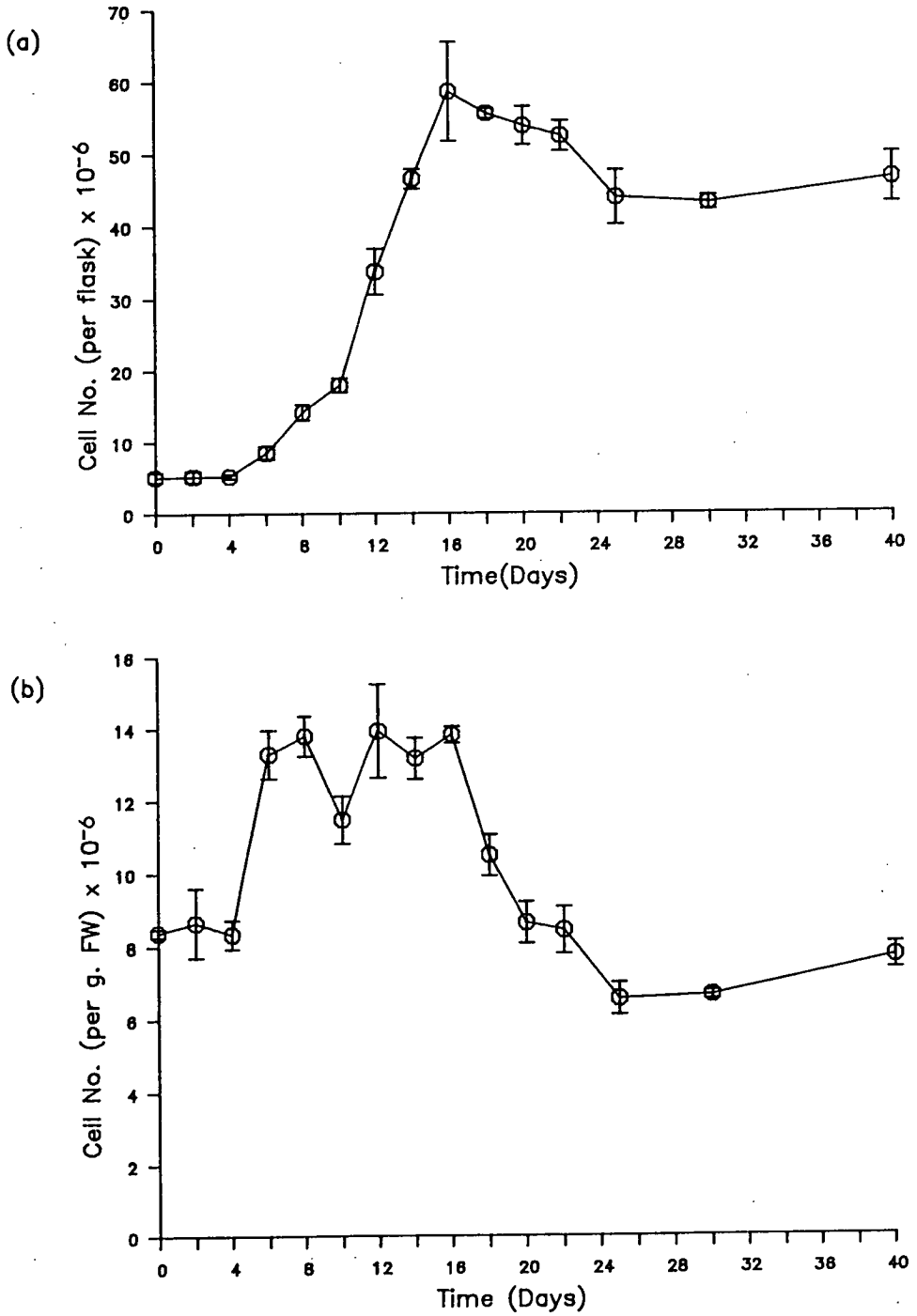
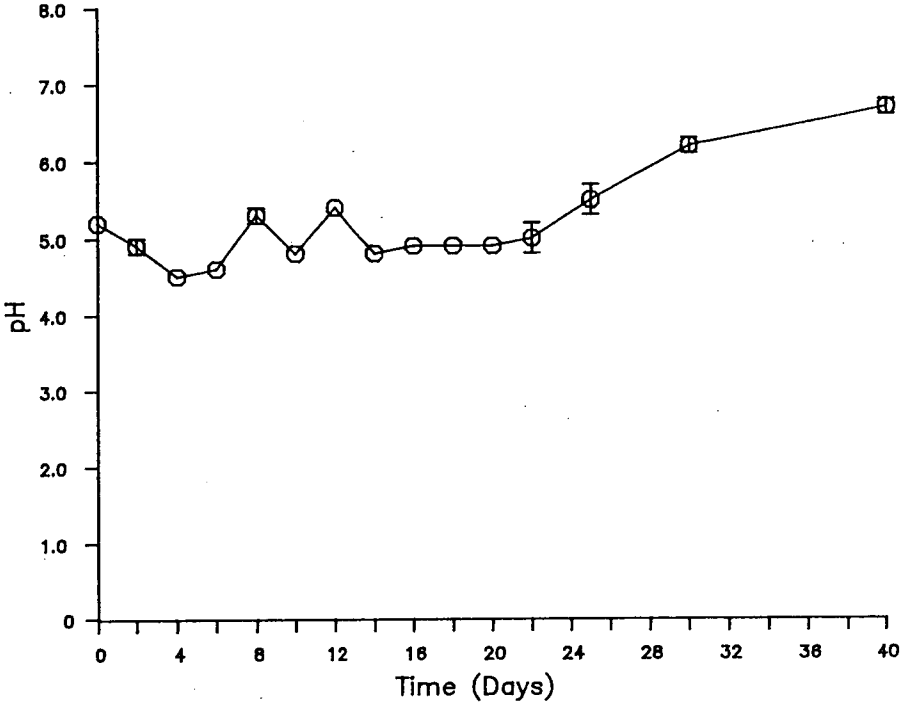


Figure 3.2.5

Changes in the pH of the culture medium of *B.orellana* suspension cultures during batch growth. Each value is the mean of three replicates ± s.e.



Summary of the Results in Section 3.2

The following points have arisen from the results reported in this section.

(1) Callus cultures of *B.orellana* were established and the growth and appearance of the cultures was improved by altering the growth regulators in the culture medium.

(2) Suspension cultures of *B.orellana* were also established and the optimum inoculation density for initiation and growth of these cultures was determined.

(3) The growth of *B.orellana* suspension cultures was characterised and shown to consist of a lag phase of *ca.* 4d. followed by cell division and growth then the stationary phase was reached by d.20.

(4) Orange pigmented cells were observed in some of the *B.orellana* callus cultures.

Having successfully established both callus and suspension cultures of *B.orellana* and found orange pigment in callus cultures this pigment was further investigated in future experiments. In the next series of experiments attempts were made to increase the amount of pigment in firstly the callus cultures and subsequently in suspended cells.

3.3 EXPERIMENTS TO IMPROVE PIGMENTATION IN CALLUS CULTURES

In the previous section (3.2) it was observed that cells with orange and yellow pigments were present in callus cultures of *B.orellana.* However, the pigment levels were very low and the amount of callus available for extraction was small so the pigment could not be measured. In this series of experiments attempts were made to improve the pigment level in callus cultures by:

(1) Alterations to the culture medium by the addition of picloram, a reduction in the NAA concentration, the removal of inorganic nitrogen and/or inorganic phosphate.

(2) Selection of populations of pigmented cells using cell-aggregate cloning and filter paper raft-nurse culture techniques.

3.3.1 The Effect of Added Picloram on the Pigmentation of Callus Cultures

Previously orange pigment was observed in callus cultures of *B.orellana.* The aim of this experiment was to improve the level of pigment by the addition of picloram to the culture medium. Picloram is a herbicide which has been reported to increase the synthesis and accumulation of carotenoid pigments and alter their composition in callus cultures of *Allium cepa* (Musker *et al.*, 1988). In this experiment a range of picloram concentrations were tested with callus of *B.orellana.*

Five different media were used: MS medium (control) (see 2.2.1.2), MS with 0.01mg.l.$^{-1}$ picloram, MS with 0.1mg.l.$^{-1}$ picloram, MS with 1mg.l.$^{-1}$ picloram and MS with 10mg.l.$^{-1}$ picloram. A known weight of callus (*ca.* 300mg.) was subcultured onto 15ml. agar medium in 5cm. Petri dishes. The callus had previously been grown on MS medium (2.2.1.2). Five replicate samples were prepared for each medium. The Petri dishes were sealed with parafilm and maintained under standard culture conditions (see 2.2.3.2). After 14d. the callus was weighed aseptically (2.3.1.1) and the viability was determined as described in 2.3.1.5. The callus was then subcultured onto fresh agar medium. Once again *ca.* 300mg. of callus was added to each Petri dish. The fresh weight and the viability were determined after a further 14d. The pigment levels in the callus were very low and there was a limited amount of callus available for extraction. Therefore, the pigment could not be measured and only visible changes in the pigment were noted. The mean relative growth rates were determined from the fresh weights as described in 2.3.1.6. The results are presented

in Table 3.3.1.

The results show (Table 3.3.1(a)) that for the first subculture the mean relative growth rate with 0.1mg.l.$^{-1}$ picloram was not significantly different to the control. However, the growth rates for the remaining treatments were all significantly less than the control for the first subculture. For the second subculture the mean relative growth rates for all of the treatments were significantly less than the control. The negative value obtained with 10mg.l.$^{-1}$ picloram in the second subculture was due to the fresh weight of the callus decreasing during the culture period. The viability of the callus was less than the control for all of the treatments for both subcultures (Table 3.3.1(b)). Therefore, it would appear from these results that apart for 0.1mg.l.$^{-1}$ picloram, during the first subculture, the addition of picloram to the medium resulted in a decrease in the mean relative growth rate of the callus compared to the control. In addition picloram also decreased the viability of the cultures as compared to the control.

The callus turned a grey/brown colour when picloram was added to the medium but there was no visible sign of pigment in any of the cultures. It would appear that picloram decreased growth and viability but did not stimulate the part of secondary metabolism necessary to promote carotenoid synthesis and accumulation. In the next experiment a further attempt was made to increase the pigment in callus cultures by decreasing the concentration of NAA in the culture medium.

Table 3.3.1(a)

Mean relative growth rates for callus cultures on five different media during two subcultures. Each value is the mean of five replicates ± s.e.

Medium	Mean Relative Growth Rate ($d.^{-1} \times 10^2$)	
	First Subculture	**Second Subculture**
control	5.70 ± 0.18	7.09 ± 0.54
MS + 0.01mg.l.$^{-1}$ picloram	3.22 ± 0.80 *	4.85 ± 0.69 *
MS + 0.1mg.l.$^{-1}$ picloram	4.80 ± 0.61	3.82 ± 0.69 **
MS + 1.0mg.l.$^{-1}$ picloram	3.62 ± 0.47 **	0.83 ± 0.17 ***
MS + 10mg.l.$^{-1}$ picloram	1.76 ± 0.56 ***	-0.37 ± 0.18 ***

(significant from the control at * P=0.05, ** P=0.01 and *** P=0.001)

Table 3.3.1(b)

Viability of callus cultures on five different media during two subcultures.

Medium	Viability (%)	
	First Subculture	**Second Subculture**
control	84	89
MS + 0.01mg.l.$^{-1}$ picloram	61	43
MS + 0.1mg.l.$^{-1}$ picloram	64	44
MS + 1.0mg.l.$^{-1}$ picloram	53	18
MS + 10mg.l.$^{-1}$ picloram	36	0

3.3.2 The Effect of Reducing the Concentration of NAA on the Pigmentation of Callus Cultures

In the previous experiment (3.3.1) it was found that the addition of picloram to the culture medium decreased the growth and viability of *B.orellana* callus cultures and there was no visible increase in the pigment in these cultures. The aim of this experiment was to study the effect of reducing the auxin (NAA) concentration in the culture medium on the pigment content of the cultures. There are reports where maximum yields of secondary metabolites were obtained when cells were grown on media devoid of auxin (see Mantell and Smith, 1983).

A known weight of callus (*ca.* 300mg.), previously grown on MS medium, was subcultured onto fresh agar medium as described in 3.3.1. In this experiment three different media were used: MS medium with 5.4 x 10^{-6}M NAA (control) (see 2.2.1.2), MS with 2.7 x 10^{-6}M NAA, which is 50% less NAA than in the control (MS50%NAA) and MS lacking NAA (MS-NAA). Five replicates were prepared for each medium. The Petri dishes were sealed and maintained under standard culture conditions (see 2.2.3.2). After 14d. the callus was weighed (2.3.1.1) and then was subcultured onto fresh agar medium. At each subculture every Petri dish contained *ca.* 300mg. of callus. The cultures were left for a further 14d. then the fresh weight and viability were measured (see 2.3.1.1 and 2.3.1.5). Once again, only visible changes in the pigment were noted because the pigment could not be measured due to the reasons outlined in 3.3.1. The mean relative growth rates were determined for the two subcultures as described in 2.3.1.6 and the results are presented in Table 3.3.2.

The mean relative growth rate (Table 3.3.2(a)) and the viability (Table 3.3.2(b)) of the callus decreased in the absence of NAA. However, on MS50%NAA the growth rate was not significantly different from the control for the two subcultures but the viability of cells on MS50%NAA was less than the control at the end of the second subculture. No visible increase in the pigmentation of the callus was observed with any of the treatments. Once again, as with the picloram, the problem appears to be that when the growth of the culture is reduced the viability is also reduced. In order to increase the level of secondary metabolites in the cultures the viability of the cultures should have been maintained while the growth decreased. In the next experiment attempts were made to achieve this by depleting the medium of certain major constituents such as nitrogen and phosphate.

Table 3.3.2(a)

Mean relative growth rates for callus cultures on three different media during two successive subcultures. Each value is the mean of five replicates ± s.e.

Medium	Mean Relative Growth Rate ($d.^{-1} \times 10^2$)	
	First Subculture	**Second Subculture**
control	8.26 ± 0.51	7.42 ± 0.38
MS50%NAA	8.26 ± 1.04	7.30 ± 0.63
MS-NAA	5.86 ± 0.98 *	4.84 ± 0.16 ***

(significant from the control at * P=0.05 and *** P=0.001)

Table 3.3.2(b)

Viability of callus cultures after two successive subcultures on three different media.

Medium	Viability After Two Subcultures (%)
control	77
MS50%NAA	66
MS-NAA	43

(The initial viability of callus cultures was 80%)

3.3.3 The Effect of Nutrient Stress on the Pigmentation of Callus Cultures

In the last two experiments (3.3.1 and 3.3.2) the addition of picloram to the medium or the reduction or removal of NAA did not appear to increase the pigment level in callus cultures. Indeed, both these treatments decreased the growth and the viability of the cultures. The aim of this experiment was to determine if the pigment level could be increased in callus cultures by the removal of two macronutrients; inorganic nitrogen (i-N) and inorganic phosphate (i-P).

There are many reports in the literature which describe how modifications to culture conditions can enhance the yields of secondary metabolites in plant tissue cultures (eg. Mantell and Smith, 1983). Previously the limitation of i-P (Knobloch and Berlin, 1981; Yamakawa *et al.*, 1983; Lindsey, 1985), i-N (Amorim *et al.*, 1977; Yamakawa *et al.*, 1983; Lindsey, 1985) and i-N + i-P (Lindsey, 1985; Hall and Yeoman, 1986b) have been shown to increase yields of secondary metabolites from plant tissue cultures.

Four different media were used in this experiment: MS medium (control), MS lacking i-P (MS-P), MS lacking i-N (MS-N) and MS lacking i-N and i-P (MS-NP) (see 2.2.1.2 and 2.2.1.5). At time 0, *ca.* 300mg. of callus was subcultured onto fresh agar medium as described previously (3.3.1). Five replicate samples were prepared for each medium. The Petri dishes were sealed and maintained as described in 2.2.3.2. After 14d. the callus was weighed and the viability was determined (see 2.3.1.1 and 2.3.1.5). The callus (*ca.* 300mg.) was then subcultured onto fresh agar medium and left for a further 14d. when the fresh weight and viability were once again measured. Only visible changes in the pigment were noted due to the reasons outlined in 3.3.1. From the fresh weights of the callus the mean relative growth rates were determined (see 2.3.1.6). The results are presented in Table 3.3.3.

The results show that all of the treatments decreased both the growth (Table 3.3.3(a)) and viability (Table 3.3.3(b)) of the callus cultures compared to the control. The mean relative growth rate and the viability of the control remained constant over the two subcultures but the treatments decreased growth and viability from the first to the second subculture. Again, similar to the previous two experiments (3.3.1 and 3.3.2), there was no visible increase in the pigment in any of the cultures. This is what would be expected since the viability of the cultures decreased for all of the treatments.

Altering the culture medium did not increase the pigment in this and the previous

two experiments (3.3.1 and 3.3.2). This is because in all three experiments the treatments decreased the growth of the cultures and also decreased the viability. To increase secondary metabolite production in the cultures the viability should have been maintained while the growth decreased. An alternative approach to increase the pigment in callus cultures was tested in the next experiment. This involved two methods which selected for pigmented cells.

Table 3.3.3(a)

Mean relative growth rates for callus cultures on four different media during two successive subcultures. Each value is the mean of five replicates ± s.e.

Medium	**Mean Relative Growth Rate ($d.^{-1} \times 10^2$)**	
	First Subculture	**Second Subculture**
control	8.02 ± 0.88	8.27 ± 1.39
MS-N	5.59 ± 0.21 *	1.29 ± 0.13 **
MS-P	3.91 ± 0.46 **	1.71 ± 0.25 **
MS-NP	4.16 ± 0.16 **	1.87 ± 0.14 **

(significant from the control at * P=0.05 and ** P=0.01)

Table 3.3.3(b)

Viability of callus cultures of *B.orellana* on four different media during two successive subcultures.

Medium	Viability (%)	
	First Subculture	Second Subculture
control	76	72
MS-N	57	28
MS-P	39	19
MS-NP	50	24

3.3.4 The Use of Selection Methods to Obtain Pigmented Callus Cultures

Previously clumps of pigmented cells were observed in callus cultures of *B.orellana*. However, experiments to increase this population of pigmented cells have been unsuccessful (3.3.1, 3.3.2 and 3.3.3). The aim of this experiment was to select for the pigmented cells. Two different techniques were used.

(a) In the first method cell-aggregate cloning was used (Yamada and Fujita, 1983). Here, the clumps of pigmented cells were removed aseptically from the callus and subcultured onto 15ml. fresh agar MS medium in 5cm. Petri dishes which were then sealed with parafilm and left for 14d. under standard culture conditions (2.2.3.2). After 14d. the pigmented callus was selected, removed and subcultured again onto fresh medium. This procedure was repeated for four subcultures and visible changes in the pigment were recorded.

(b) In the second method the filter paper raft-nurse culture technique as described by Muir *et al.* (1954) was used. Pieces of established callus were subcultured onto MS agar medium (15ml.) in 5cm. Petri dishes then a sterile 8x8mm. square of filter paper was placed aseptically on top of each piece of callus. The Petri dishes were sealed with parafilm and maintained under standard culture conditions (2.2.3.2).

After 2-3d. a small piece of pigmented callus was placed on the filter paper raft. The cultures were left for 14d. then the filter paper was transferred to a fresh piece of host callus on MS medium. This step was necessary because as the host culture became old and senescent the young culture it supported also stopped growing. Once the cultures on the filter paper were large enough (4mm. diameter) they were transferred directly onto agar medium where they grew independently. This technique supplies by diffusion through the paper barrier not only all the essential nutrients of the medium capable of supporting the growth of the callus but those extra factors necessary to induce cell division. Once again visible changes in the pigment of the callus were recorded.

With the first method, the callus either died, because the pieces of tissue were too small, or the callus grew but consisted predominantly of non-pigmented cells. With successive subcultures the callus became less pigmented. A similar result was obtained using the filter paper raft-nurse technique. The pieces of callus grew but they had more non-pigmented than pigmented cells. It would appear from these results that there was a decrease in the pigment as reflected by the number of coloured cells as the callus grows. However, the fate of the pigment in the cultures cannot be determined from these results.

Summary of the Results in Section 3.3

The following points have emerged from the experiments described in this section.

(1) Picloram (a herbicide), when added to the culture medium, was found to decrease the growth and viability of the cultures but it did not stimulate the production of carotenoid pigments.

(2) Reducing the concentration of NAA in the culture medium did not increase the pigment in callus cultures.

(3) Removing two macronutrients (i-N and i-P) decreased the growth and viability of the callus cultures but it did not increase the pigment in these cultures.

(4) Two selection methods (cell-aggregate cloning and filter paper raft-nurse culture technique) which selected for the pigmented cells also did not increase the pigment in callus cultures of *B.orellana.*

In these experiments attempts were made to increase the pigment in callus cultures so that it could be measured and thus investigated. However, the pigment was not increased by any of these methods and the fate of the pigment during culture was not determined. Accordingly, in the next section changes in the carotenoid pigment levels during callus initiation were measured in an alternative approach to investigate the fate of the carotenoid pigments.

3.4 CHANGES IN PIGMENT LEVELS DURING CALLUS INITIATION FROM ROOT TISSUE

Orange pigment is present at low concentration in cultures of *B.orellana*, however, the pigment disappears as the cultures age. In the last series of experiments (3.3) attempts were made to promote pigment production in callus cultures but these were not successful. Therefore, an alternative approach was tried in order to determine the fate of this pigment during culture. This approach involved measuring the changes in carotenoid pigment levels during callus initiation.

Explants from the primary roots of seedlings were chosen as an experimental system because :

(a) Roots have previously been shown to have pigmented cells (3.1).

(b) Root tissue from seedlings can be obtained quickly and easily.

(c) Sterile primary root tissue is easily obtained by germinating seeds under sterile conditions.

(d) A more uniform explant can be obtained from the primary root tissue of seedlings.

(e) There is no chlorophyll present which makes analysis straightforward.

(f) Callus can be initiated routinely from seedling root tissue.

3.4.1 Measurement of the Changes in Total Carotenoid Pigment During Callus Initiation from Root Tissue

The aim of this experiment was to determine the fate of the endogenous carotenoid pigment during callus initiation from root explants.

Explants (transverse sections, 4mm. long) were cut from the primary root of 3-4d. old sterile seedlings (2.2.2.3 and 2.2.1.4). Seedlings with a radicle between 2-3cm. were chosen from within the population to ensure uniformity (see Fig. 3.4.1). The terminal 1.5cm. of the root was first removed then the extreme tip containing the meristem was excised and discarded. The remaining part of the root (1cm.) was then placed in a sterile Petri dish and the dish placed over a piece of graph paper to enable the 4mm. explants to be cut. 4mm. was found to be the minimum amount of tissue required to initiate callus. Two explants were obtained from each root and both were

used because they had similar pigmentation and were found to initiate callus equally well. Single explants were weighed then placed on 15ml. of standard agar growth medium (2.2.1.2) in sterile 5cm. Petri dishes. Each Petri dish was sealed with parafilm and placed in standard culture conditions (2.2.3.2). Twelve Petri dishes were harvested every four days until and including d.40. Carotenoid pigment was extracted from nine explants in groups of three as described in 2.3.3.1 and 2.3.3.3 to ensure that the amount of pigment could be calculated. These explants were weighed prior to extraction.

A typical spectrum obtained in this experiment is shown in Fig. 3.4.2. This is for total carotenoid pigment extracted so there is more than one pigment present. It shows that there is an absorption maximum at 420nm. In order to compare the pigment in different samples a comparison of the absorption maxima at 420nm. was made. The proportion of pigmented cells was determined in each of the remaining three explants as described in 2.3.3.4. The results are presented in Figs. 3.4.3, 3.4.4 and 3.4.5. The appearance of the explants on d.0 and d.40 is shown in Fig. 3.4.6.

The results presented in Fig. 3.4.3 show that over 40d. there was a 17 fold increase in the fresh weight of the explants as callus tissue was initiated. Initially the increase over the first 12d. was small, but significant (at P=0.001 level), and was followed by a much larger increase between d.12 and d.28. There was no significant increase in fresh weight after d.28.

The changes in pigment levels in the explants over the culture period are shown in Fig. 3.4.4(a). A large decrease in pigment level occurred during the first four days (3 fold). This was probably due to the loss of pigment at the cut surface of the explant because when the explants were cut pigment was found to move to the cut surface. Pigment on the cut surface of the explant can be seen in Fig. 3.4.6(a). A possible explanation of this is that the pigment is mainly contained in channels at the outer edge of the cortex of the root (3.1) and cutting releases the pigment from these. After this initial decrease, the pigment increased 2.8 fold between d.4 and d.24, then remained constant until d.40.

From the data presented in Fig. 3.4.4(b) it can be seen that there was also a large decrease in pigment per mg. fresh weight of tissue during the first four days (4.6 fold). Between d.4 and d.20 there was a 1.5 fold increase in the pigment followed by a 2.3 fold decrease between d.20 and d.32. Subsequently the pigment level remained constant.

The data presented in Fig. 3.4.5 show that the proportion of pigmented cells was very low and remained constant over the whole culture period. Since the numbers of pigmented cells was so low and the s.e.s were very high accurate measurement was difficult and underlying trends could have been hidden. Variation in the tissue or experimental error or both could account for the s.e.s being so high. There is some suggestion that the number of pigmented cells increased since the proportion of pigmented cells remained constant when the total cell number increased. This increase in pigmented cells could have been occurring in the root or the newly initiated callus tissue, or both.

Although the pigment level decreased during the first four days, the fact that it increased between d.4 and d.24 shows that pigment production was occurring. Whether this pigment production was occurring in the root or in the newly initiated callus tissue is not known. In the next experiment the root and callus tissue were extracted and analysed separately to establish this point.

Figure 3.4.1

The appearance of a 3-4d. old seedling of *B.orellana* from which root explants were excised.

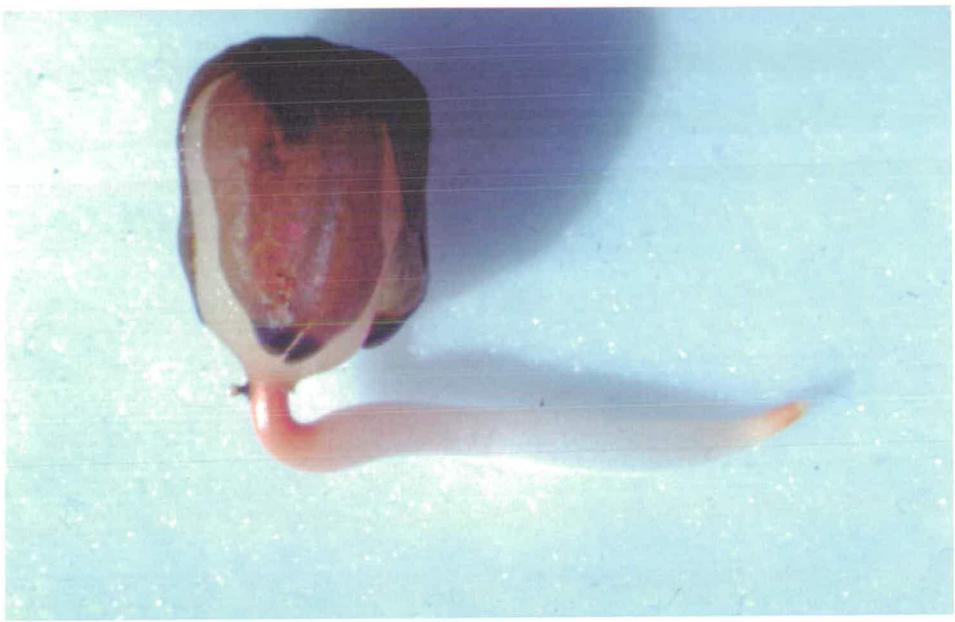


Figure 3.4.2

A typical spectrum obtained for the total carotenoid pigment extracted in experiments described in 3.4.1, 3.4.2 and 3.4.3.

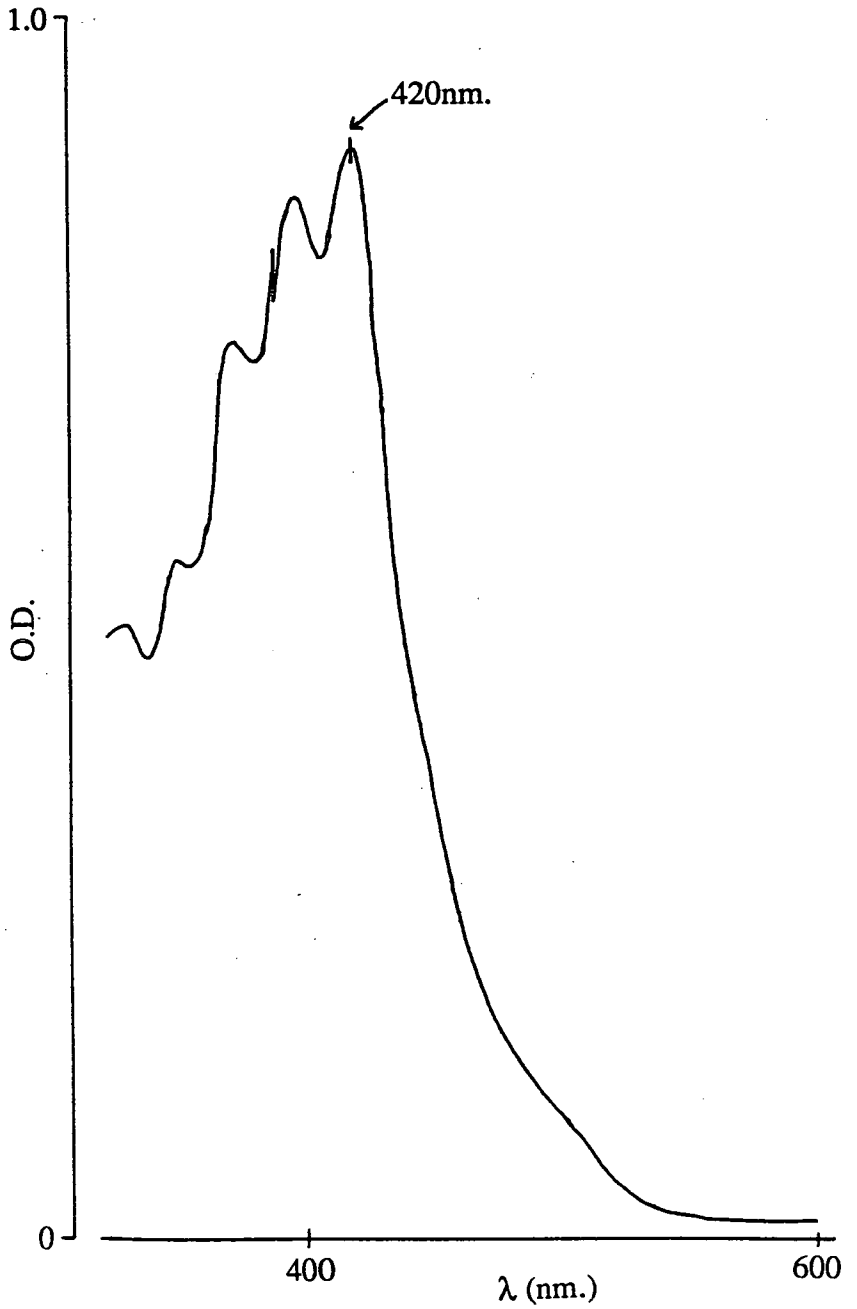


Figure 3.4.3

Changes in the fresh weight of root explants of *B.orellana* during callus initiation. Each value is the mean of three replicates ± s.e.

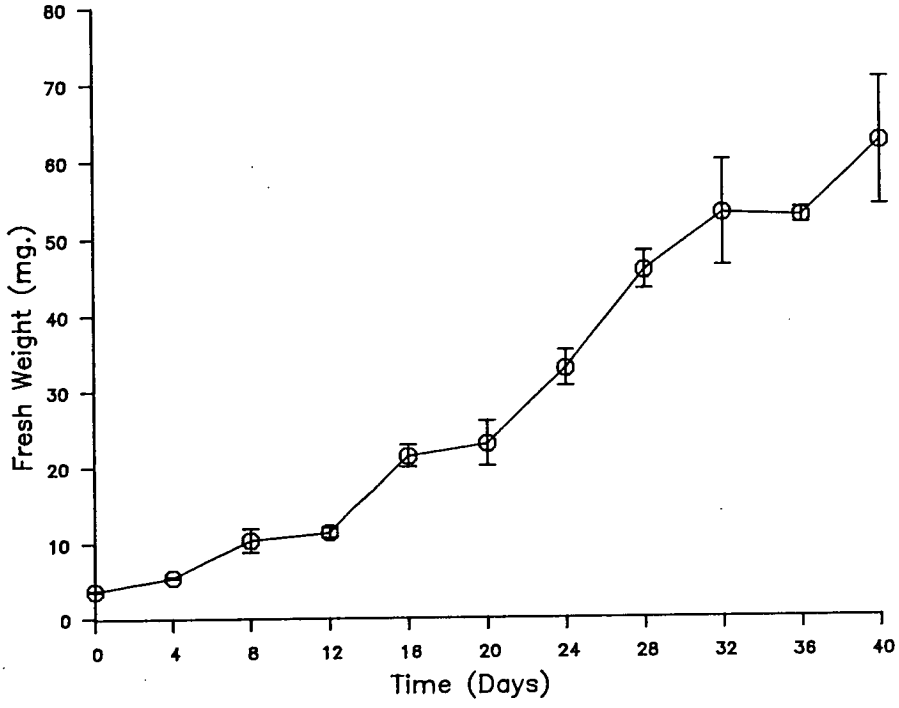


Figure 3.4.4

Changes in total carotenoid pigment per explant (a) and per mg. fresh weight (b) measured at 420nm. in root explants of *B.orellana* during callus initiation. Each value is the mean of three replicates ± s.e.

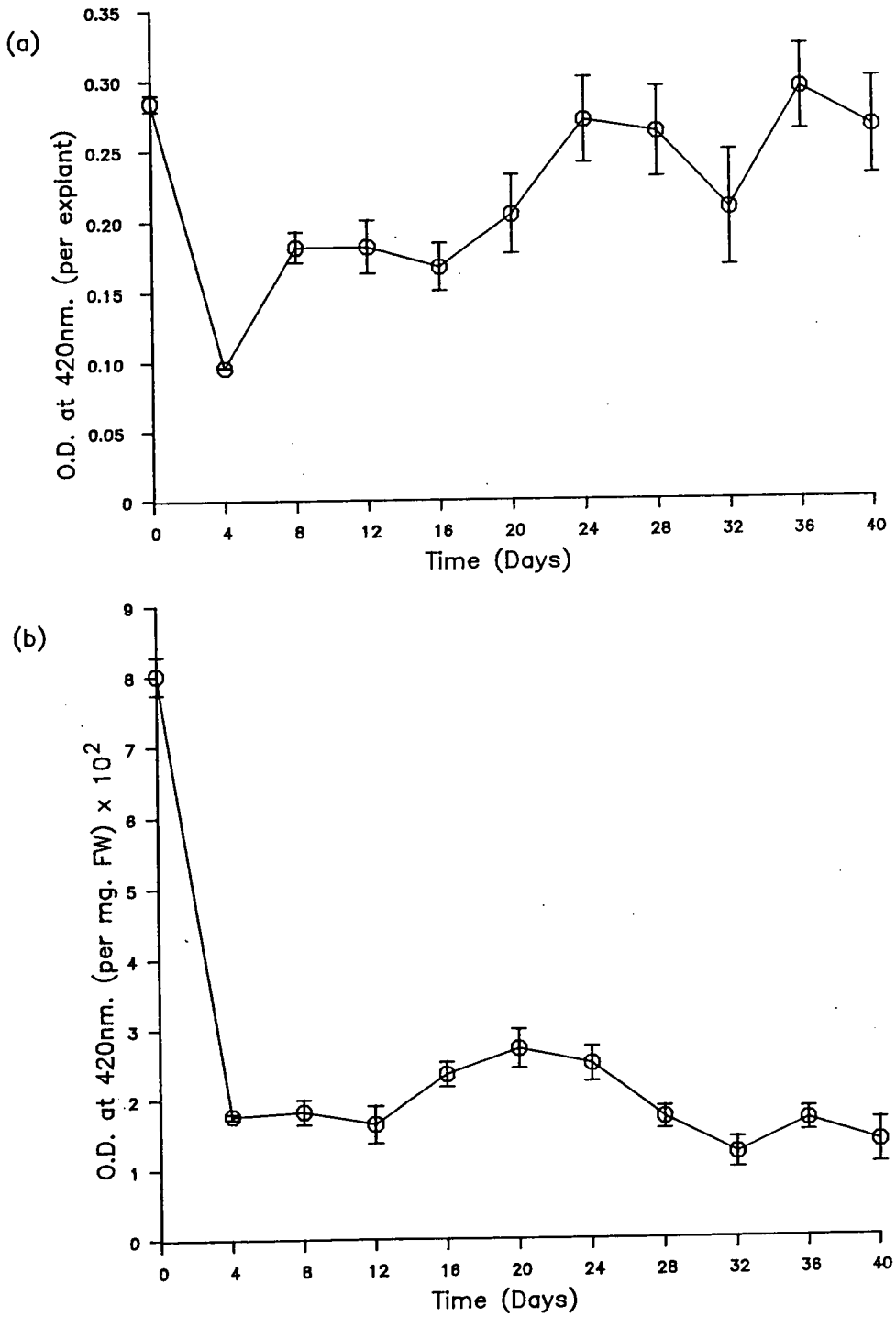


Figure 3.4.5

Changes in the proportion of pigmented cells in root explants of *B.orellana* during callus initiation. Each value is the mean of three replicates ± s.e.

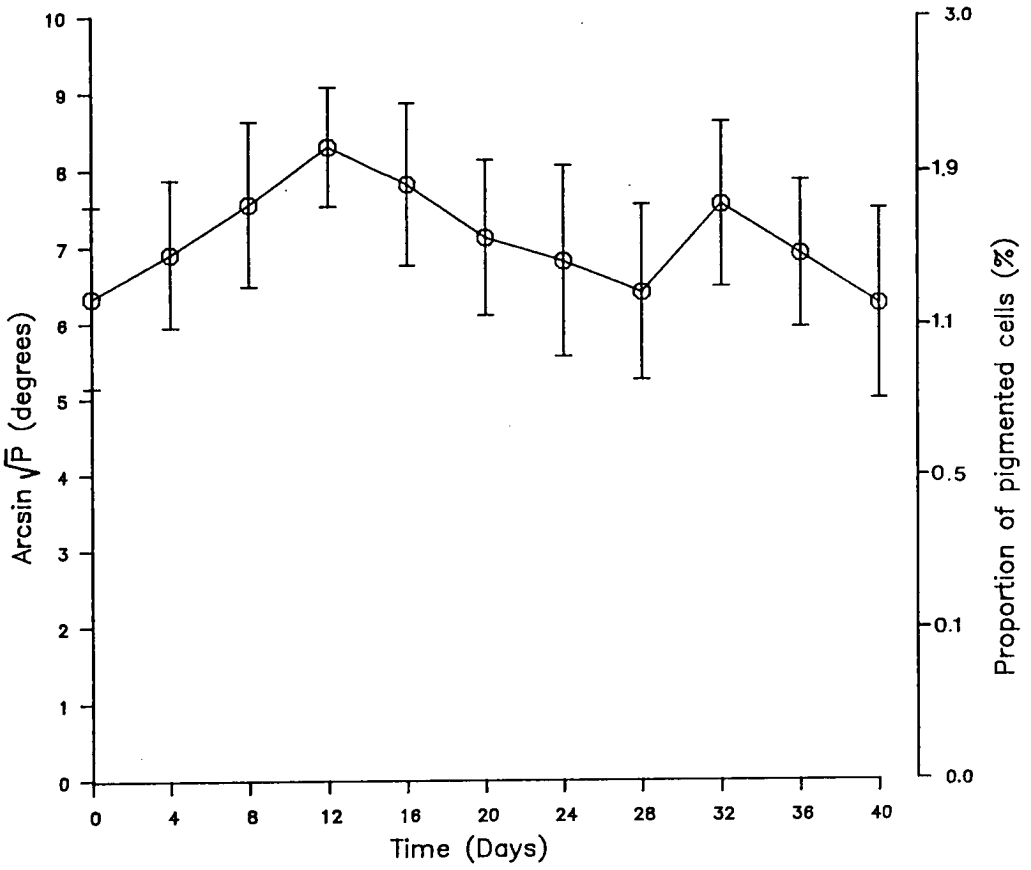
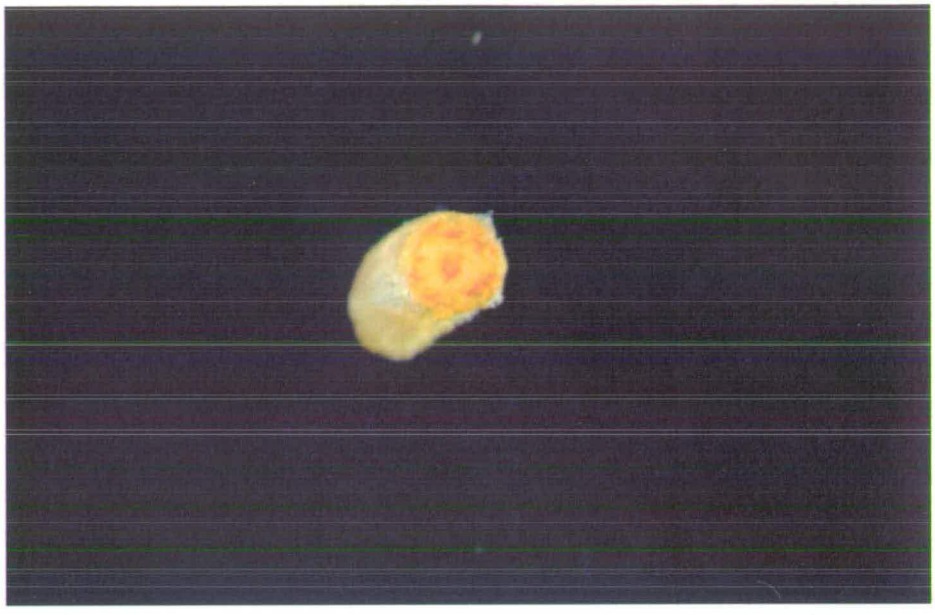
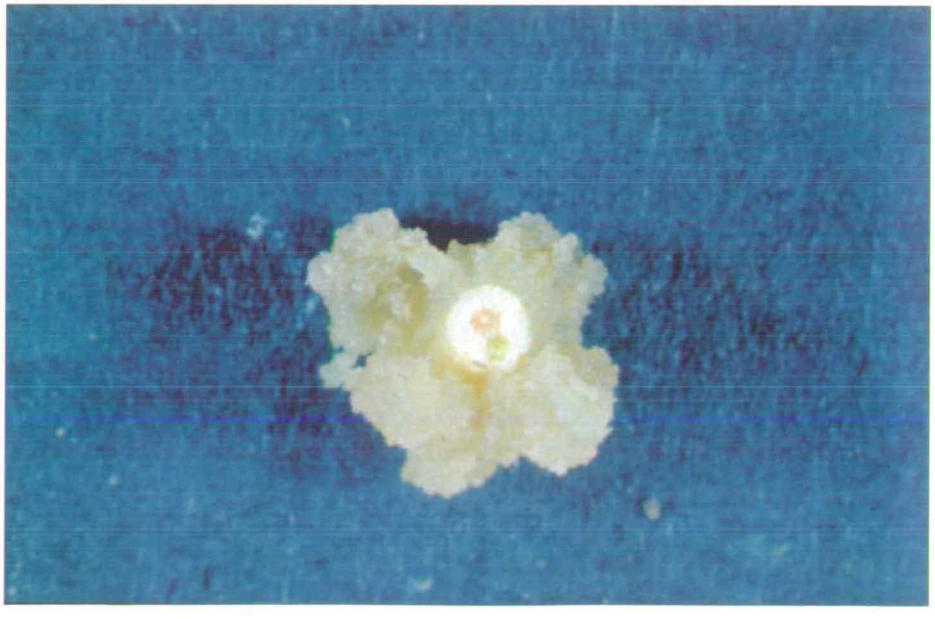


Figure 3.4.6

(a) A root explant of *B.orellana* on d.0 of the callus initiation period, illustrating pigment on the cut surface of the explant.



(b) A root explant of *B.orellana* on d.40 of the callus initiation period.



3.4.2 Measurement of the Changes in Carotenoid Pigment in Root and Callus Tissue During Callus Initiation from Root Tissue

In the last experiment an increase in the carotenoid pigment level was observed during callus initiation from root explants. However, it was not known where this increase was occurring. Therefore, the aim of this experiment was to determine whether the increase was taking place in the root or newly initiated callus tissue. The approach was similar to the previous experiment but root and callus tissue were analysed separately.

Root explants were prepared and callus was initiated as described in the previous experiment (3.4.1). On days 0, 4, 16, 20, 24, 28 and 40 fifteen Petri dishes, each containing one explant, were harvested. Carotenoid pigment was extracted separately from the root and newly initiated callus tissue of nine of the explants in groups of three as described in 2.3.3.1 and 2.3.3.3 to ensure that the amount of pigment could be calculated. The root and callus tissue were weighed prior to extraction. In three of the explants the root and callus tissue were weighed then the cell number was determined as described in 2.3.1.3. Using the remaining three explants, the proportion of pigmented cells was estimated in the root and callus tissue as described in 2.3.3.4. The results are presented in Figs. 3.4.7, 3.4.8, 3.4.9 and 3.4.10.

The increase in fresh weight of the explants was mostly due to the formation of callus tissue (Fig. 3.4.7). There was a 13 fold increase in the fresh weight of the callus tissue (based on the d.0 root explant weight as the initial value) but only a 3 fold increase in the root fresh weight over 40d. On d.0 and d.4 no callus tissue had formed.

From the data presented in Fig. 3.4.8(a) it can be seen that there was more pigment per explant in the root than in the newly initiated callus tissue (*ca.* 3 fold). In the root tissue there was a decrease in the pigment in the first four days similar to that found in the previous experiment (3.4.1). The values on d.0 and d.4 for callus tissue were zero because no callus had formed. Between d.4 and d.20 there was a 2.6 fold increase in pigment in the root explant and the callus increased from zero to an O.D. of *ca.* 0.1. After d.20 the pigment in both the root and callus tissue remained constant.

Changes in pigment levels per mg. fresh weight of tissue are shown in Fig.3.4.8(b). Once again there was a greater amount of pigment in the root than in the callus (*ca.* 10 fold). Also the amount of pigment in the root decreased sharply

between d.0 and d.4 (4.7 fold) whereas the amount in callus tissue was zero. Between d.4 and d.20 there was a 1.9 fold increase in the pigment per mg. fresh weight in the root and the callus increased from zero to an O.D. of *ca.* 4×10^{-3}. The pigment then decreased 2 fold for the root and 4.1 fold for the callus between d.20 and d.40.

The data presented in Fig. 3.4.9(a) show that initially the cell number per explant was greater for the root tissue but after d.24 the values were higher for callus. The root cell number per explant increased 4.9 fold between d.0 and d.16 then remained constant. For the callus tissue the cell number was zero on d.0 and d.4 as no callus had been formed subsequently the callus cell number increased until the end of the experiment.

From the results presented in Fig. 3.4.9(b) it can be seen that there were more cells per mg. fresh weight in the root than in the callus (*ca.* 1.5 fold). This shows that the root cells were smaller than the callus cells. Between d.0 and d.4 there was no significant increase in the cell number per mg. fresh weight in either the root or callus tissue. The cell number increased 2.1 fold between d.4 and d.16 for the root tissue then remained constant. For the callus tissue the cell number increased from zero to *ca.* 9×10^3 cells between d.4 and d.20 then decreased 1.2 fold between d.20 and d.24. After d.24 the callus cell number per mg. fresh weight remained constant.

The proportion of pigmented cells was very low in both the root and callus tissue (Fig. 3.4.10) and apart from an increase in callus between d.4 and d.20 there was no significant change during the culture period. However, the values in the root were *ca.* 3 fold higher than those in the callus tissue.

Overall, the results suggest that the reason the root had more pigment than the newly initiated callus tissue was at least partly due to an increased number of pigmented cells in the root because the proportion of pigmented cells was greater in the root. The pigment increased in both the root and callus tissue during the culture period. This increase also appears to be partly due to an increase in the number of pigmented cells since the proportion of pigmented cells either increased or remained constant when the total cell number increased. An increase in the amount of pigment per cell may also have been occurring but this could not be determined from these results. In the next experiment attempts will be made to increase this pigment production by altering the culture medium. The treatments used would be expected to reduce cell growth and primary metabolism and thus promote secondary metabolism.

Figure 3.4.7

Changes in root and callus fresh weight (per explant) during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

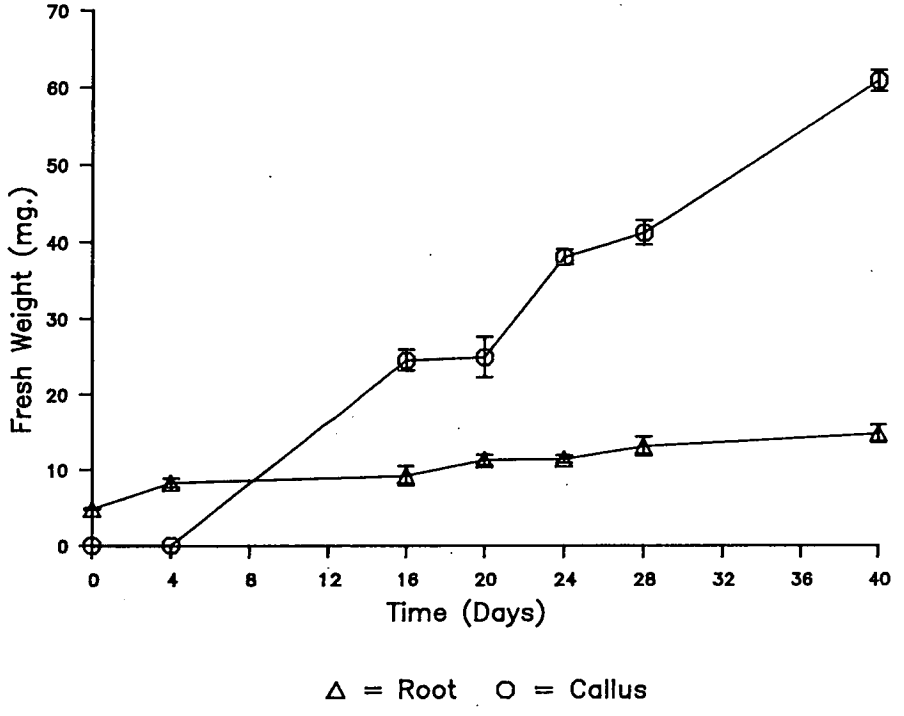


Figure 3.4.8

Changes in root and callus carotenoid pigment per explant (a) and per mg. fresh weight (b) measured at 420nm. during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

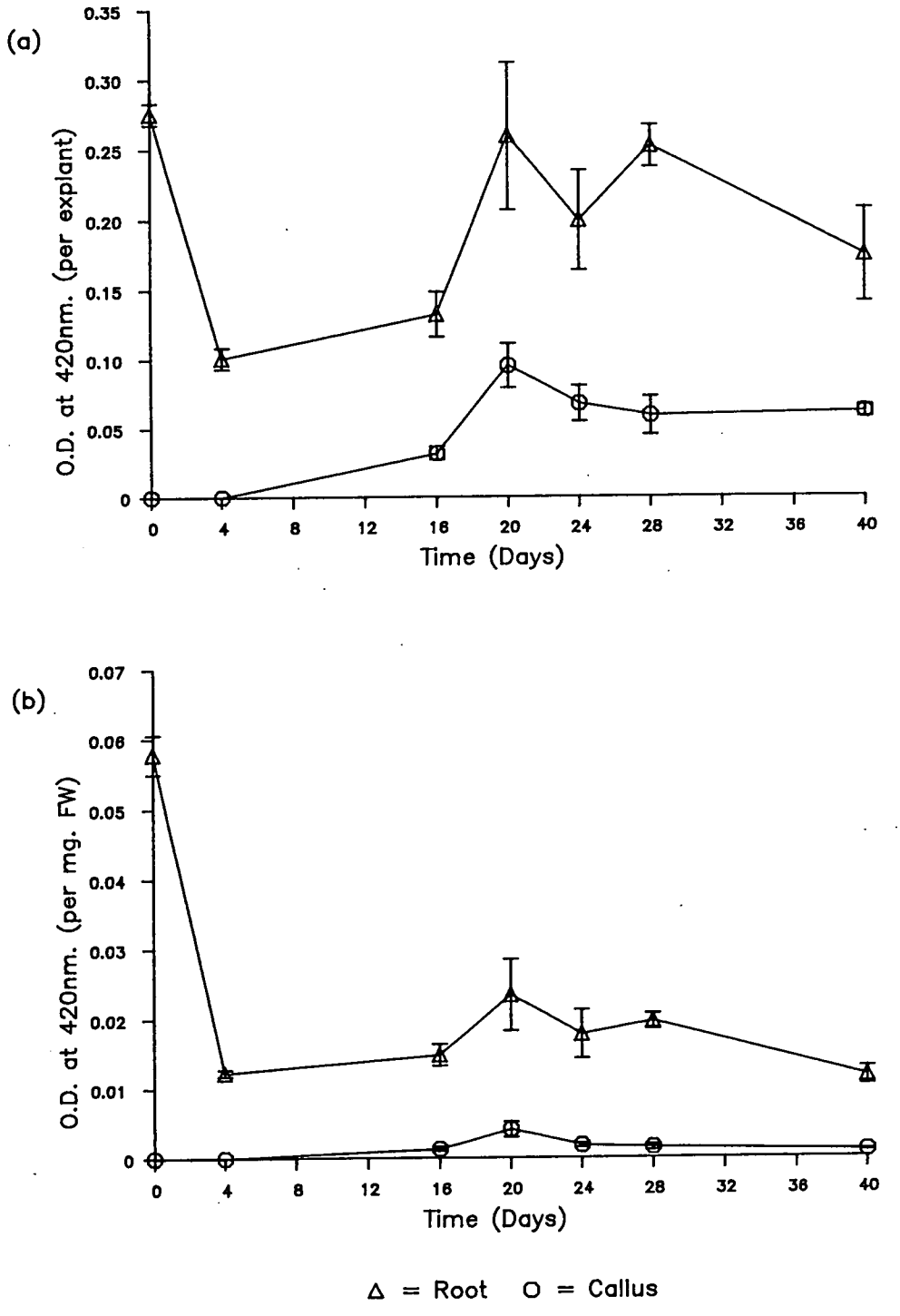


Figure 3.4.9

Changes in root and callus cell number per explant (a) and per mg. fresh weight (b) during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

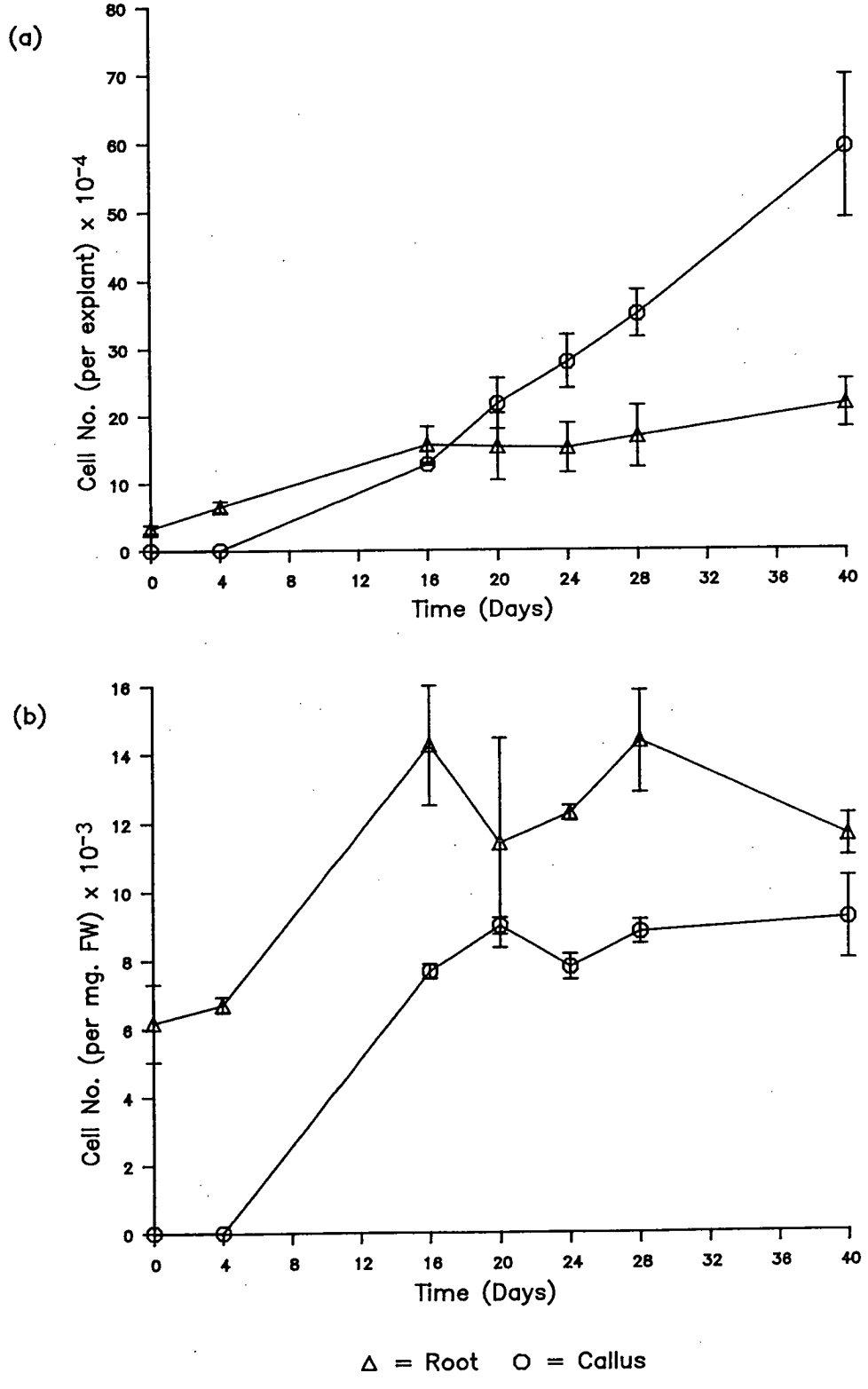
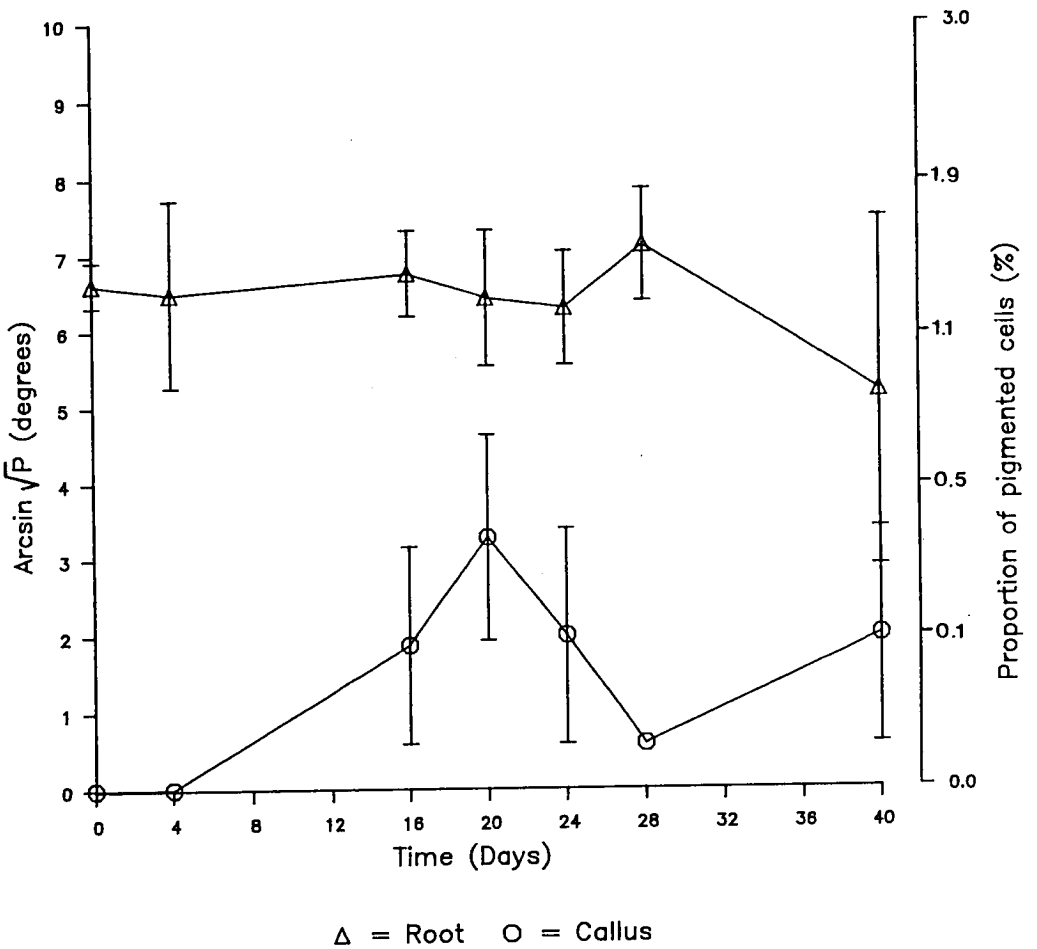


Figure 3.4.10

Changes in the proportion of pigmented cells in root and callus tissue during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.



3.4.3 Determination of the Effect of Nutrient Stress on Carotenoid Pigment Levels During Callus Initiation from Root Tissue

Previously, it was shown that both callus and root tissue displayed the ability to produce carotenoid pigment during callus initiation from root explants (see 3.4.2). This increase in pigment appeared to be partly due to an increase in the number of pigmented cells. The aim of this experiment was to discover if this pigment production could be increased by altering the culture medium.

As mentioned in 3.3.3 there are reports in the literature of limitation of inorganic nitrogen (i-N) and/or inorganic phosphate (i-P) increasing secondary metabolite production in tissue cultures. High sucrose concentrations (Knobloch and Berlin, 1980; Knobloch *et al.*, 1982; Yamakawa *et al.*, 1983) have also been found to increase secondary metabolite yields in culture.

Root explants were prepared and callus tissue was initiated as described previously (see 3.4.1). Five different media were used: MS medium (control), MS lacking in i-N (MS-N), MS lacking in i-P (MS-P), MS lacking in both i-N and i-P (MS-NP) and MS medium with 8% instead of 3% sucrose (MS8%S). These media are described in 2.2.1.2 and 2.2.1.5. On days 0, 12, 20 and 28 fifteen Petri dishes were harvested for each treatment. The carotenoid pigment, fresh weight, cell number and proportion of pigmented cells in both the root explant and the newly initiated callus tissue were estimated for each treatment as described in 3.4.2. The results are presented in Figs. 3.4.11, 3.4.12, 3.4.13, 3.4.14, 3.4.15, 3.4.16, 3.4.17, 3.4.18, 3.4.19, 3.4.20, 3.4.21, and 3.4.22. The appearance of the explants on the five different media on d.28 is shown in Fig. 3.4.23.

In the control there was an increase (2 fold) in the fresh weight of the root tissue between d.0 and d.12 after which it remained constant (Fig. 3.4.11). Compared to the control the increase in root fresh weight was less in all the other treatments. Initially on MS8%S the fresh weight increased more slowly than the control but by d.20 it was not significantly different. Both the MS-N and MS-NP media gave similar results to the control between d.0 and d.12 but the fresh weights were significantly less than the control on d.20 (at P=0.1) and d.28 (at P=0.01). The fresh weight of the root on MS-P was not significantly different from the control on d.0, d.12 and d.20, but was significantly less (at P=0.05) than the control on d.28. The callus fresh weight increased for all of the treatments over the culture period (Fig. 3.4.12). However, all of the treatments grew less than the control (MS8%S at P=0.05 on d.20, P=0.1 on d.28; MS-N at P=0.05 on d.12 and d.28, P=0.01 on d.20; MS-P at P=0.1 on d.12,

P=0.05 on d.20 and d.28; MS-NP at P=0.05 on d.12, d.20 and d.28). The largest difference in fresh weight for both the root and callus tissue was seen when the medium was lacking i-N. The increase in callus fresh weight over the culture period was greater than the root fresh weight for all of the treatments.

The results presented in Fig. 3.4.13 show that there was a small decrease in the root pigment per explant for the control over the culture period (1.3 fold). Between d.0 and d.20 the pigment levels for all of the treatments were similar to the control. On d.28 both the MS8%S and MS-N treatments had higher levels of carotenoid pigment than the control, however, only the result for MS8%S was significantly higher than the control (at P=0.05). The pigment levels on both MS-P and MS-NP were not significantly different from the control on d.28. For the control callus (Fig. 3.4.14) the pigment per explant increased until d.12 then remained constant. The callus pigment for both MS-P and MS-NP were not significantly different from the control throughout the culture period. Both MS8%S and MS-N were similar to the control between d.0 and d.12, but on d.20 they appeared to be less than the control. However, these values were found not to be significantly different from the control. Between d.20 and d.28 the pigment per explant on MS8%S increased so that it was significantly higher than the control on d.28 (at P=0.001). On d.28 the pigment level on MS-N was similar to the control. When 8% sucrose was present in the medium the pigment per explant in both the root and callus increased *ca.* 2 fold. However, this increase was not significant until d.28. Overall there was less pigment in the callus than in the root tissue.

The data presented in Fig. 3.4.15 show that root pigment per mg. fresh weight for the control decreased sharply until d.12 then remained constant. When i-P was absent from the medium the pigment levels were similar to the control. However, on MS-N, MS-NP and MS8%S the pigment per mg. fresh weight in the root was higher than the control on d.20 and d.28. These values were only significantly higher than the control on d.28 (MS-N and MS-NP at P=0.1, MS8%S at P=0.05). For callus, the pigment per mg. fresh weight increased between d.0 and d.12 for the control then remained constant (Fig. 3.4.16). The decrease between d.20 and d.28 was not significant. On MS8%S, MS-N, MS-P and MS-NP the pigment levels were the same as the control between d.0 and d.20. The higher pigment levels than the control for MS-N and MS-NP on d.12 were not significant. On d.28 the pigment levels on these media were maintained whereas the level in the control had decreased. The result was that all four media gave significantly higher pigment levels than the control on d.28 (significant at P=0.01 for MS8%S and MS-NP; P=0.05 for MS-N; P=0.1 for

MS-P). Generally, there was less pigment per mg. fresh weight in the callus than the root.

The root cell number per explant increased 5.3 fold between d.0 and d.12 then remained constant for the control (Fig. 3.4.17). Similar results were obtained with the MS8%S medium. On MS-N, MS-P and MS-NP media the root cell number per explant was less than the control. However, it was only significantly less for all three media on d.20 (at P=0.05) and for MS-NP on d.28 (at P=0.1). For the control callus tissue the cell number per explant increased over the culture period (Fig. 3.4.18). Between d.0 and d.20 the results for MS8%S were not significantly different from the control. On d.28 the cell number for MS8%S appeared to be less than the control but this was not significant. The callus cell number per explant was less than the control on MS-N, MS-P and MS-NP media (significant at P=0.05 on d.12 for MS-N, MS-P and MS-NP; P=0.1 for MS-P on d.20; P=0.01 for MS-N and P=0.001 for MS-P and MS-NP on d.28).

Changes in the root and callus cell number per mg. fresh weight are shown in Figs. 3.4.19 and 3.4.20 respectively. For the control, in both the root and callus tissue, there was an increase between d.0 and d.12 then it remained constant. On MS8%S the root cell number was not significantly different from the control. The callus cell number on MS8%S appeared to be higher than the control, but this was found not to be significant. The cell number per mg. fresh weight for root and callus on MS-N, MS-P and MS-NP media were all lower than the control during the culture period. For the root tissue the cell number was significantly less than the control for MS-N on d.12 (at P=0.05), for MS-P on d.12 (at P=0.01) and d.20 (at P=0.05) and MS-NP on d.20 (at P=0.01). The callus cell number was significantly less than the control on d.12 and d.20 for MS-N, d.20 for MS-NP and d.12, d.20 and d.28 for MS-P (all at P=0.01). Therefore, these media appeared to promote an increase in cell size. The callus cell number per mg. fresh weight was less than the root for all of the media. This shows that the cells in the callus were larger than the root cells.

Over the 28d. period the proportion of pigmented cells in both the root and callus tissue on the control medium remained constant (Figs. 3.4.21 and 3.4.22). For the root tissue the proportion of pigmented cells was only significantly higher than the control on d.12 for the MS-N treatment (at P=0.1). The increases on d.28 for MS8%S and on d.20 for MS-N were not significant. A significant decrease in the proportion of pigmented cells compared to the control only occurred on d.20 for MS-P (at P=0.1). The decreases on d.20 for MS8%S and MS-N were not significant. The

proportion of pigmented cells in the callus for the MS-N, MS-P and MS-NP treatments were not significantly different from the control. On MS8%S the proportion appeared to be higher than the control on d.20, but this was not significant. On all of the media there were more pigmented cells in the root than the callus tissue.

It would appear that the effect of MS8%S, MS-N, MS-P and MS-NP media was to decrease the growth of both the root and callus tissue and to increase pigment production. This is what would be expected as it is widely accepted that there is an inverse relationship between growth and secondary metabolite production. However, the carotenoid pigment per explant was only significantly higher than the control on MS8%S in both the root and callus tissue (*ca.* 2 fold). The pigment increased for the root on MS-N, but this was not significant. Compared to the control, an increase in root carotenoid pigment per mg. fresh weight occurred on MS8%S (*ca.* 2.1 fold), MS-N (*ca.* 2.1 fold) and MS-NP (*ca.* 1.6 fold) media whereas all of the media increased the callus pigment per mg. fresh weight (MS8%S *ca.* 3.7 fold, MS-NP *ca.* 3.2 fold and MS-N and MS-P *ca.* 3.0 fold). Therefore, the presence of 8% sucrose appears to have the greatest effect on pigment production and lack of i-P has the least effect. A significant increase in the proportion of pigmented cells was obtained only in the root tissue and when the medium was lacking i-N.

Figure 3.4.11

Changes in root fresh weight (per explant) during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

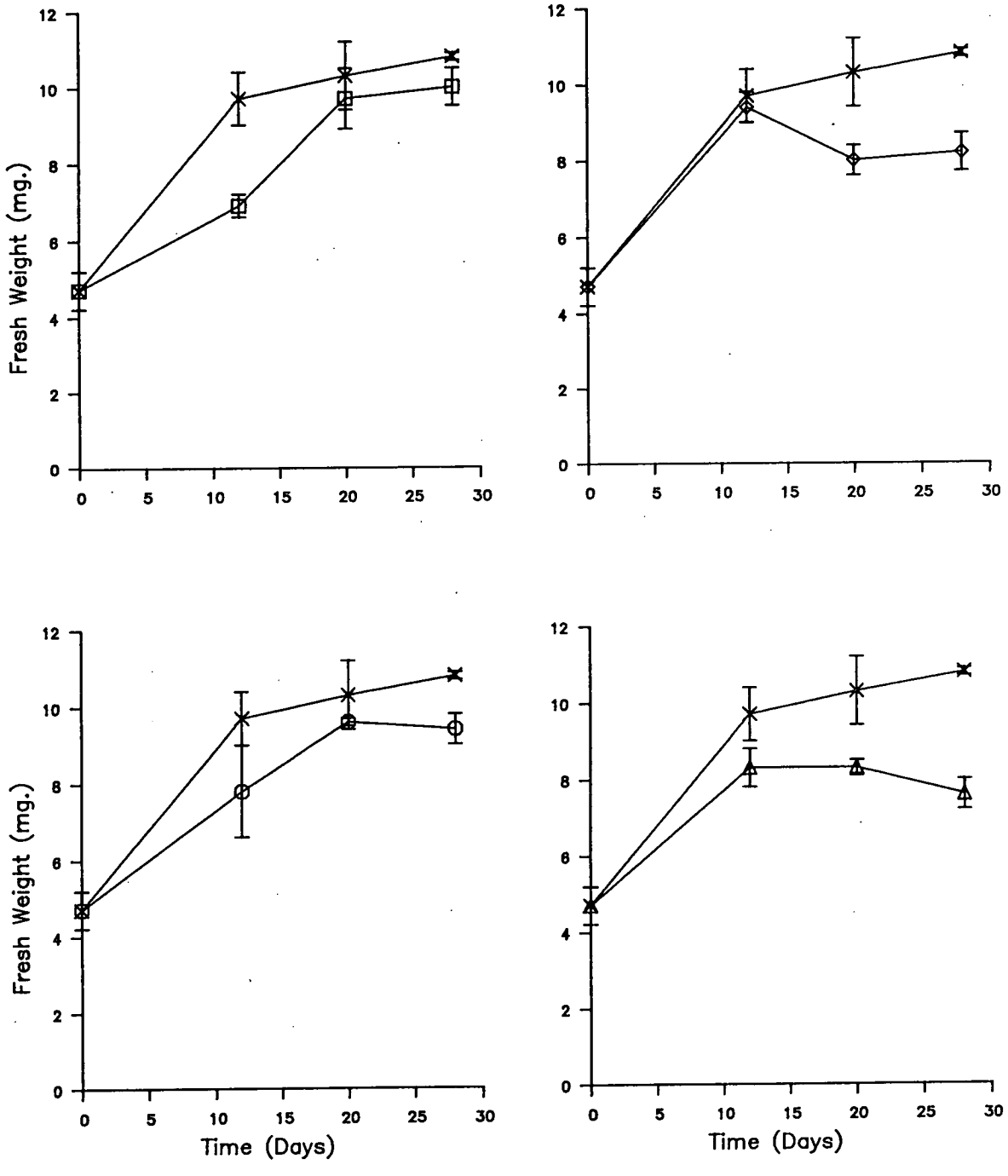


Figure 3.4.12

Changes in callus fresh weight (per explant) during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

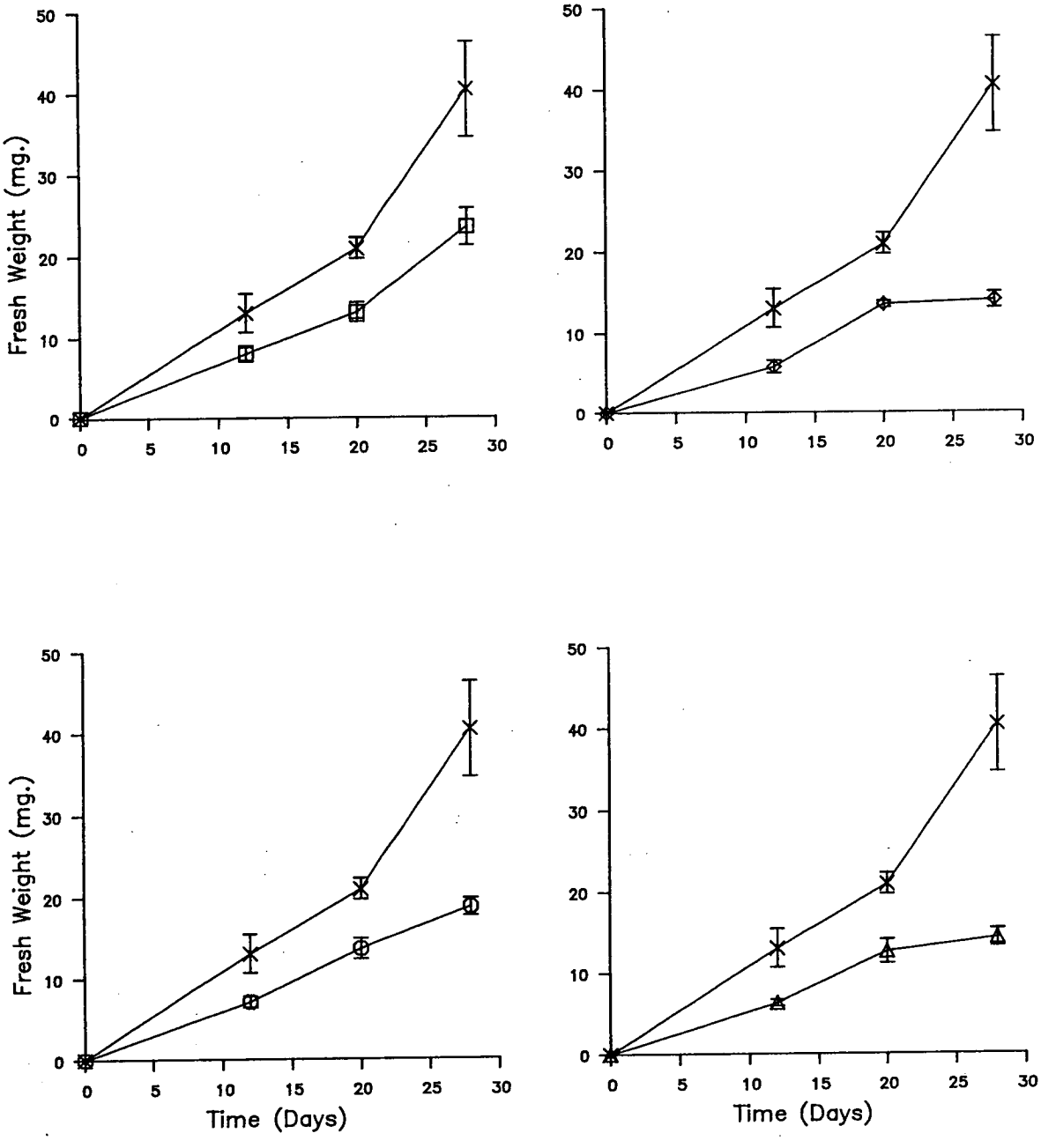


Figure 3.4.13

Changes in root carotenoid pigment (per explant) measured at 420nm. during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

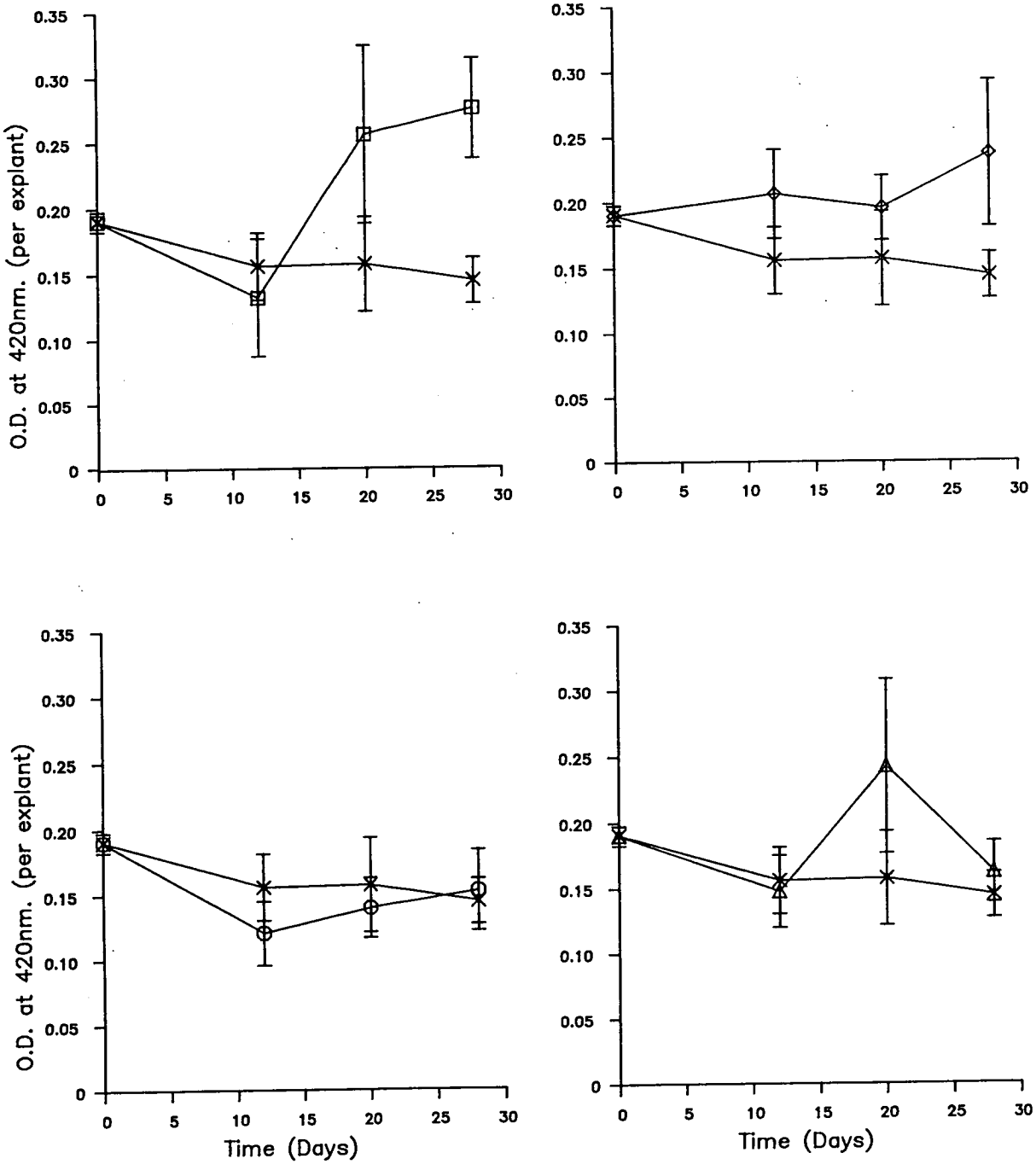


Figure 3.4.14

Changes in callus carotenoid pigment (per explant) measured at 420nm. during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

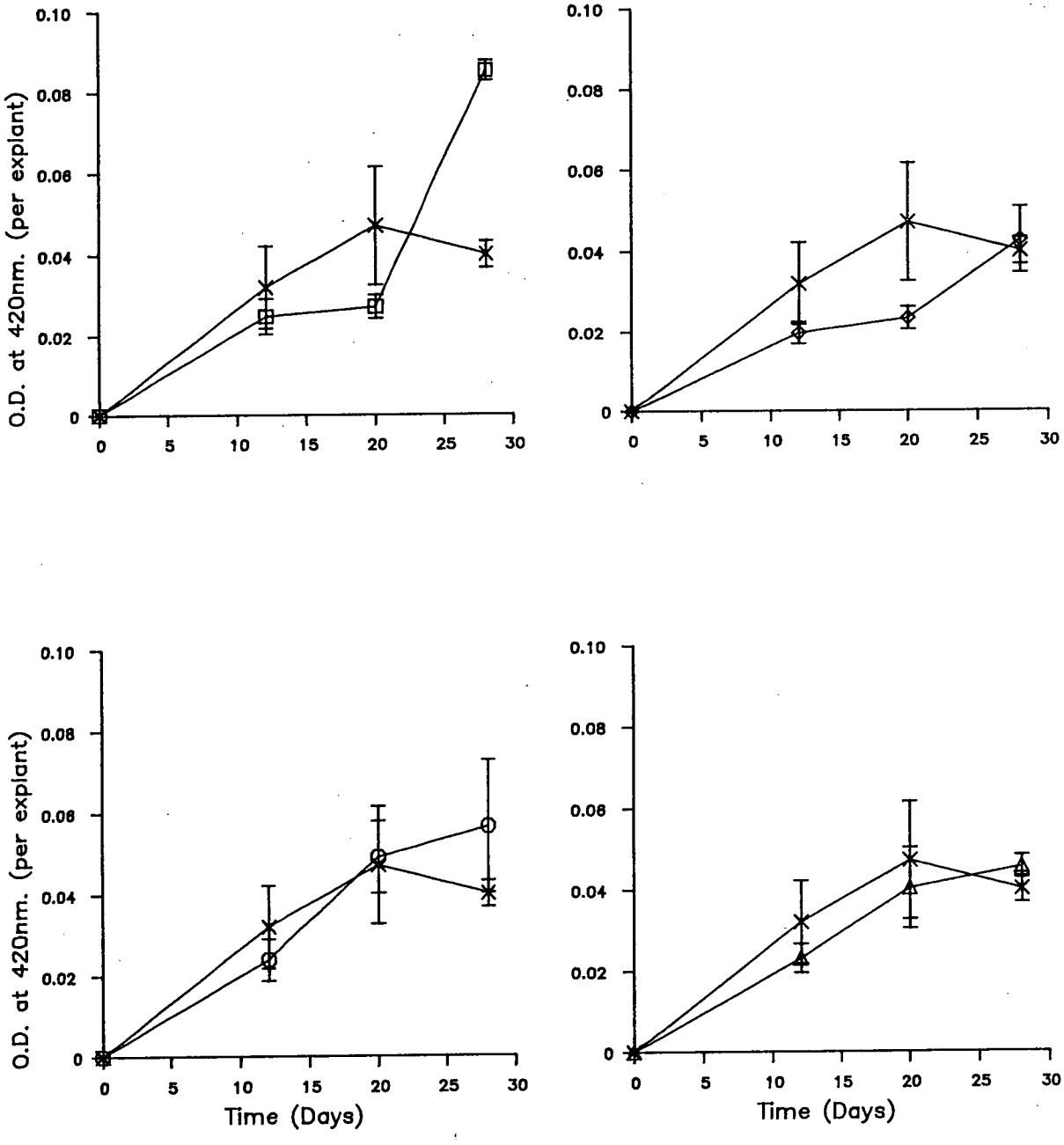


Figure 3.4.15

Changes in root carotenoid pigment (per mg. fresh weight) measured at 420nm. during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

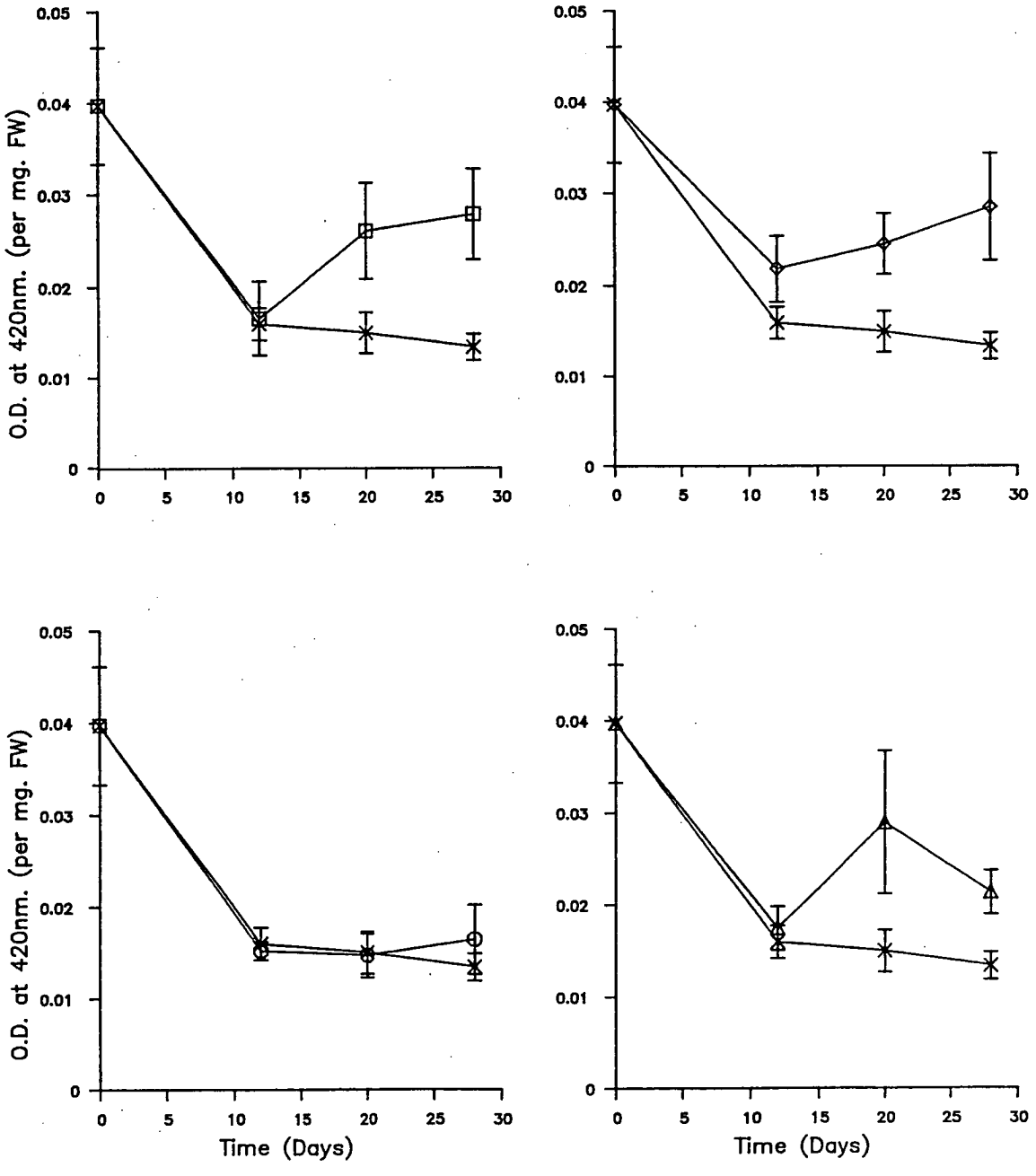


Figure 3.4.16

Changes in callus carotenoid pigment (per mg. fresh weight) measured at 420nm. during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

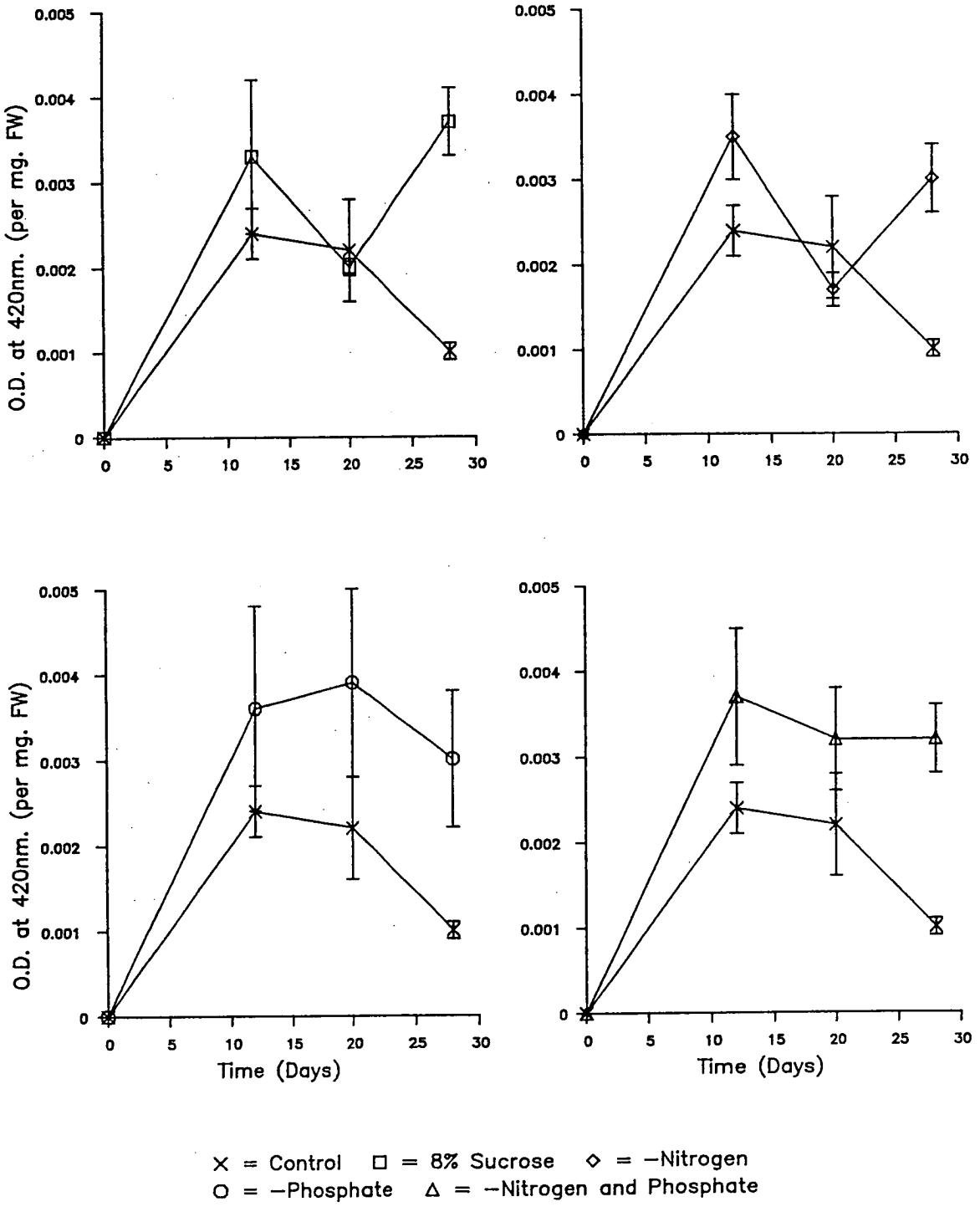


Figure 3.4.17

Changes in root cell number (per explant) during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

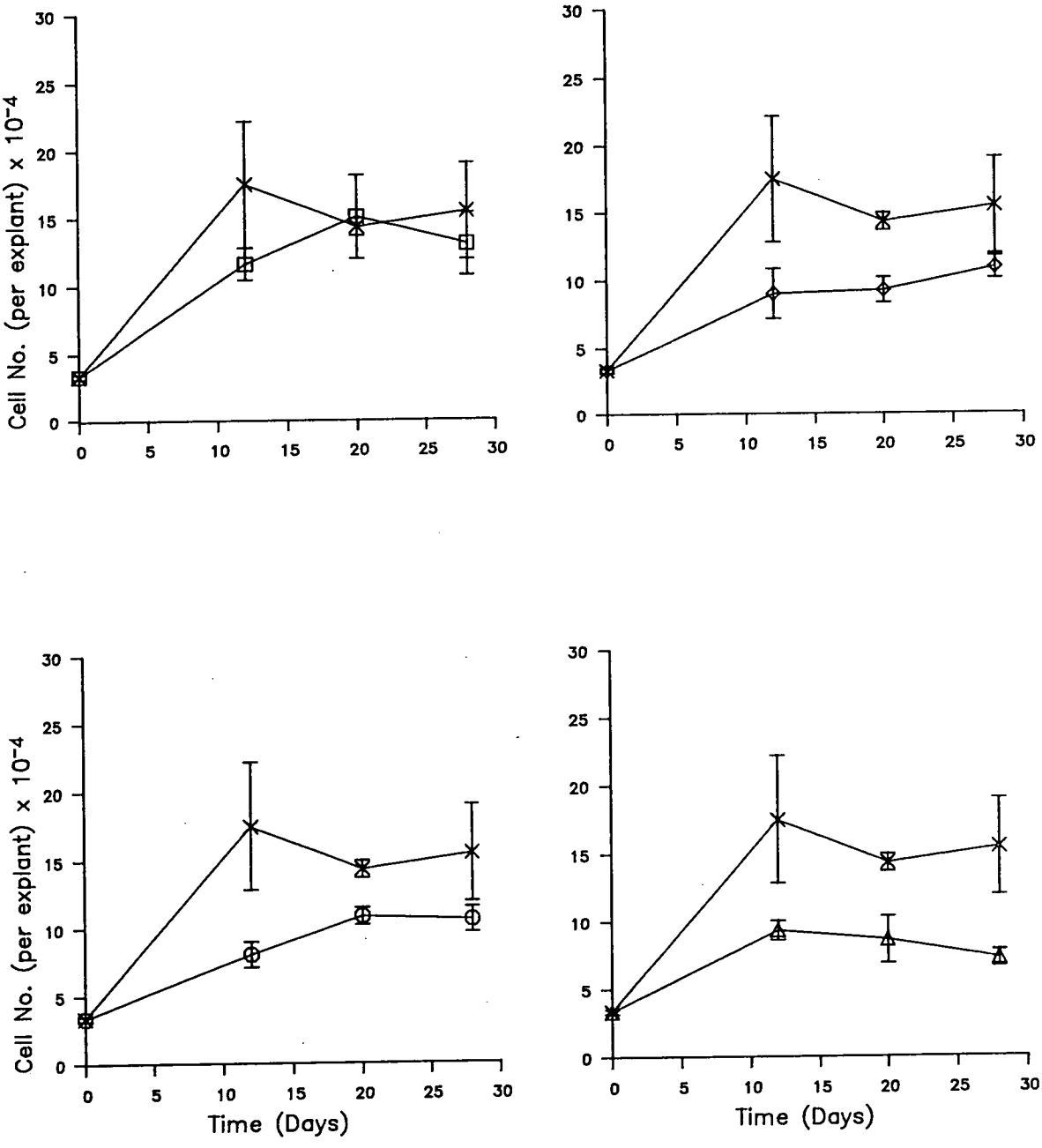


Figure 3.4.18

Changes in callus cell number (per explant) during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

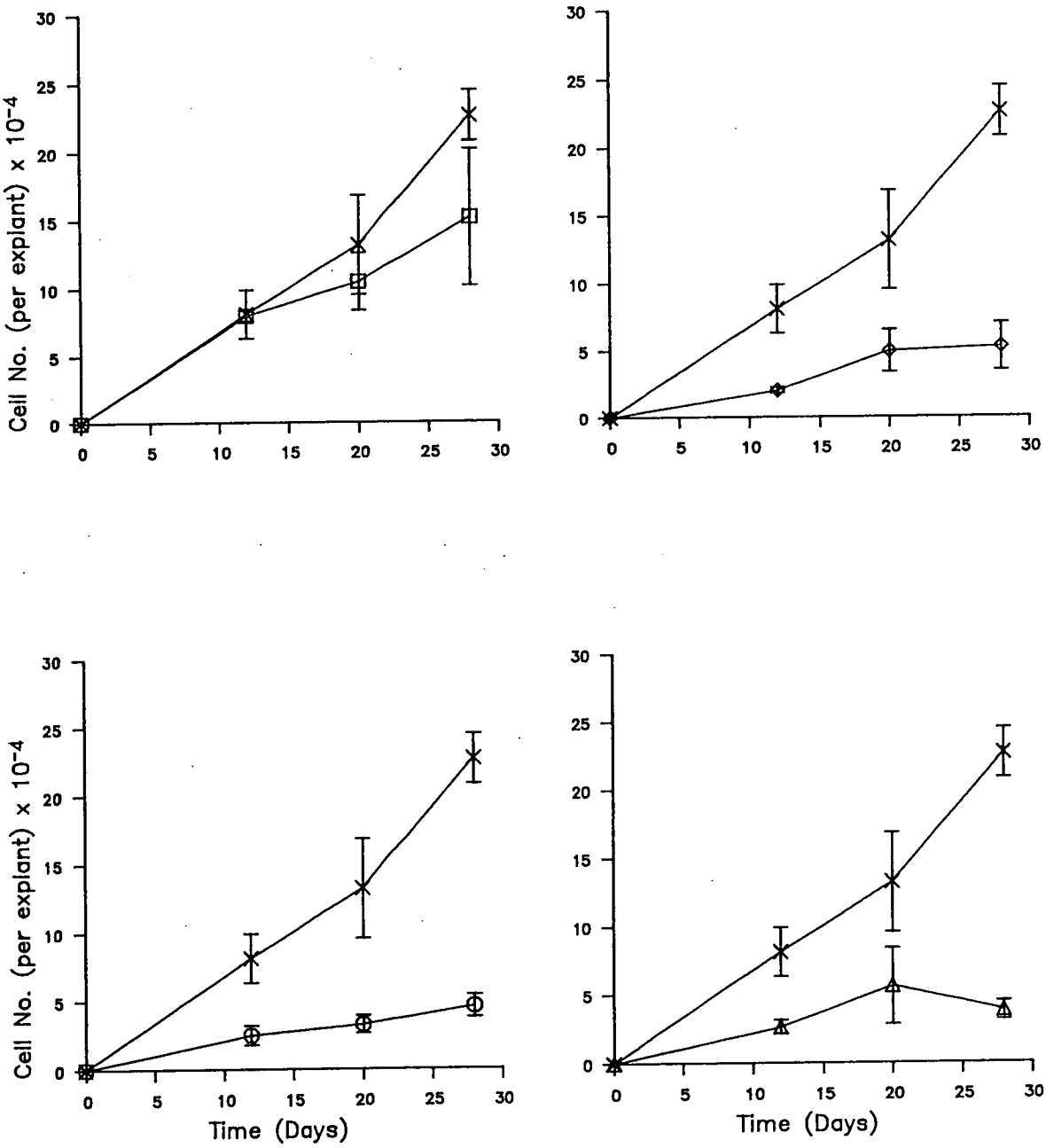


Figure 3.4.19

Changes in root cell number (per mg. fresh weight) during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

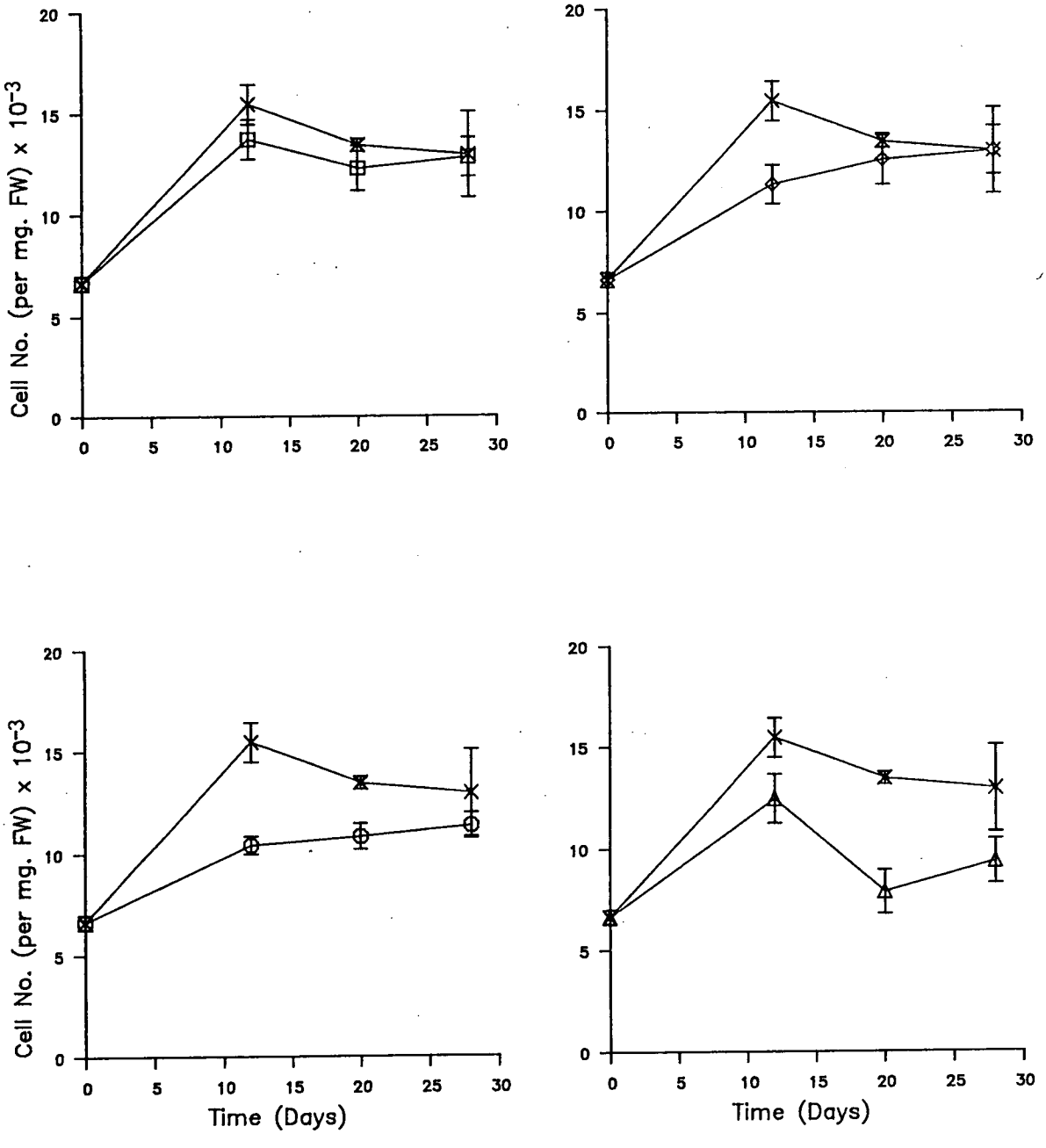


Figure 3.4.20

Changes in callus cell number (per mg. fresh weight) during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

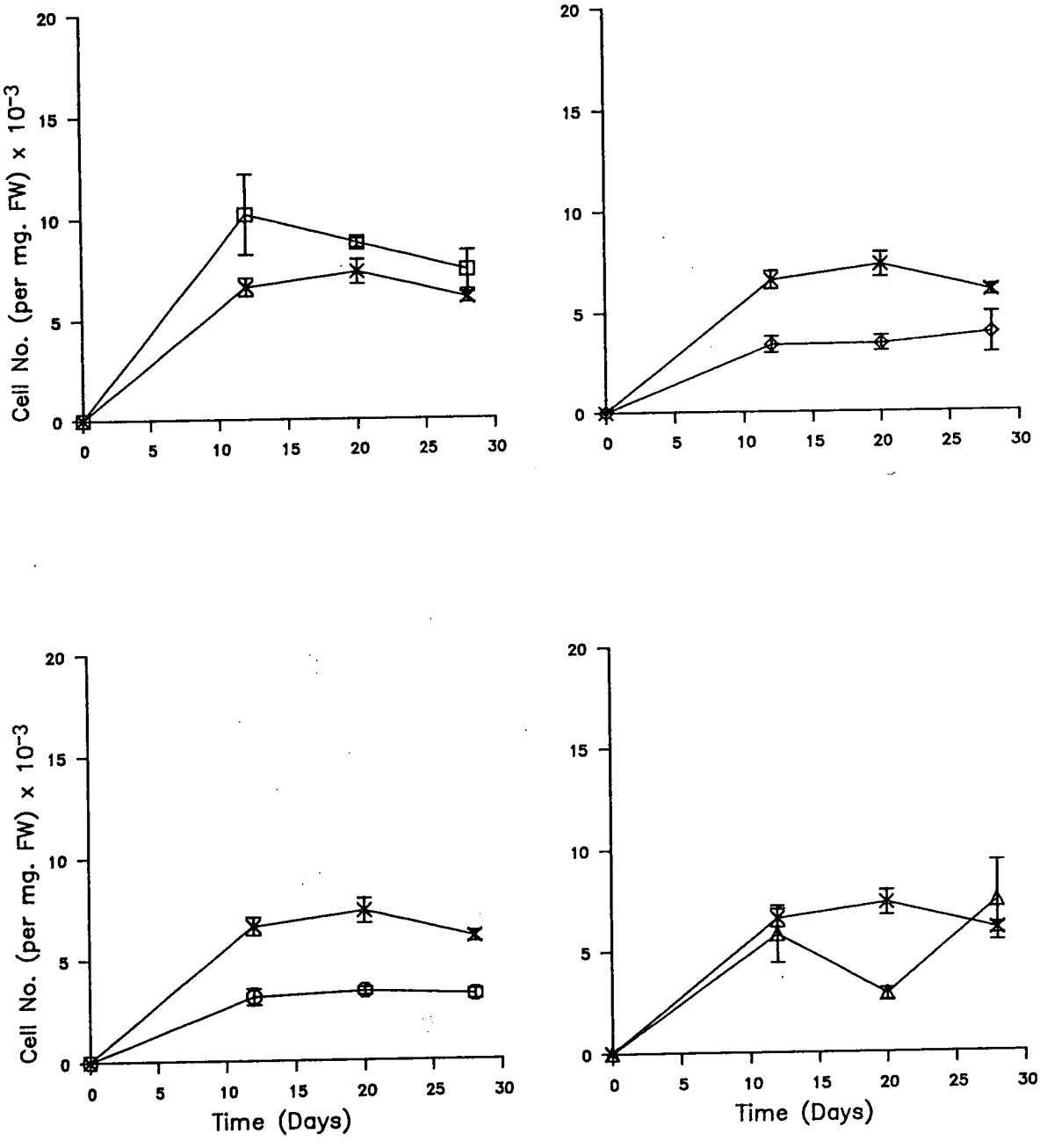


Figure 3.4.21

Changes in the proportion of pigmented cells in the root during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

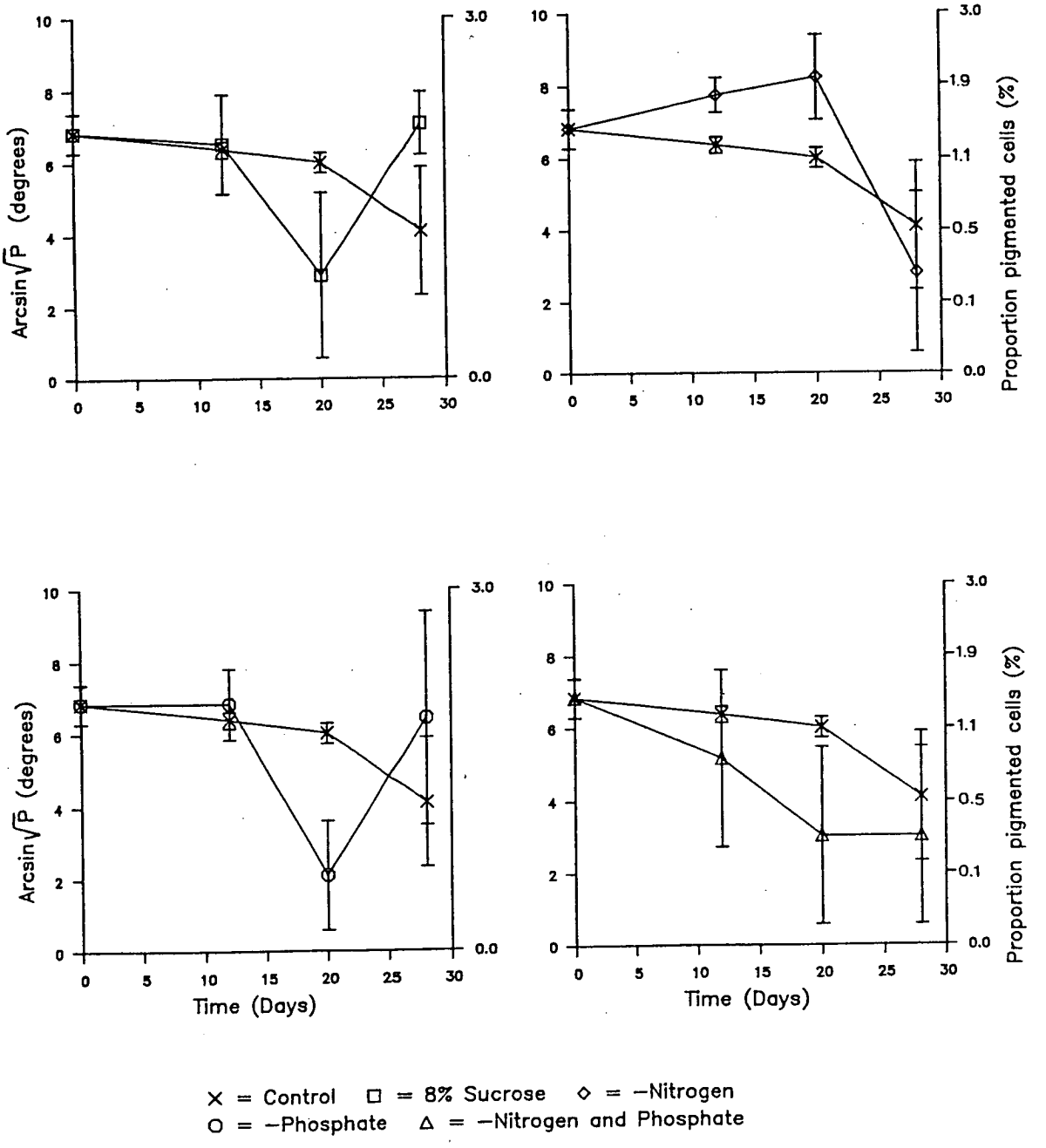


Figure 3.4.22

Changes in the proportion of pigmented cells in the callus tissue during callus initiation from root explants of *B.orellana*. Each value is the mean of three replicates ± s.e.

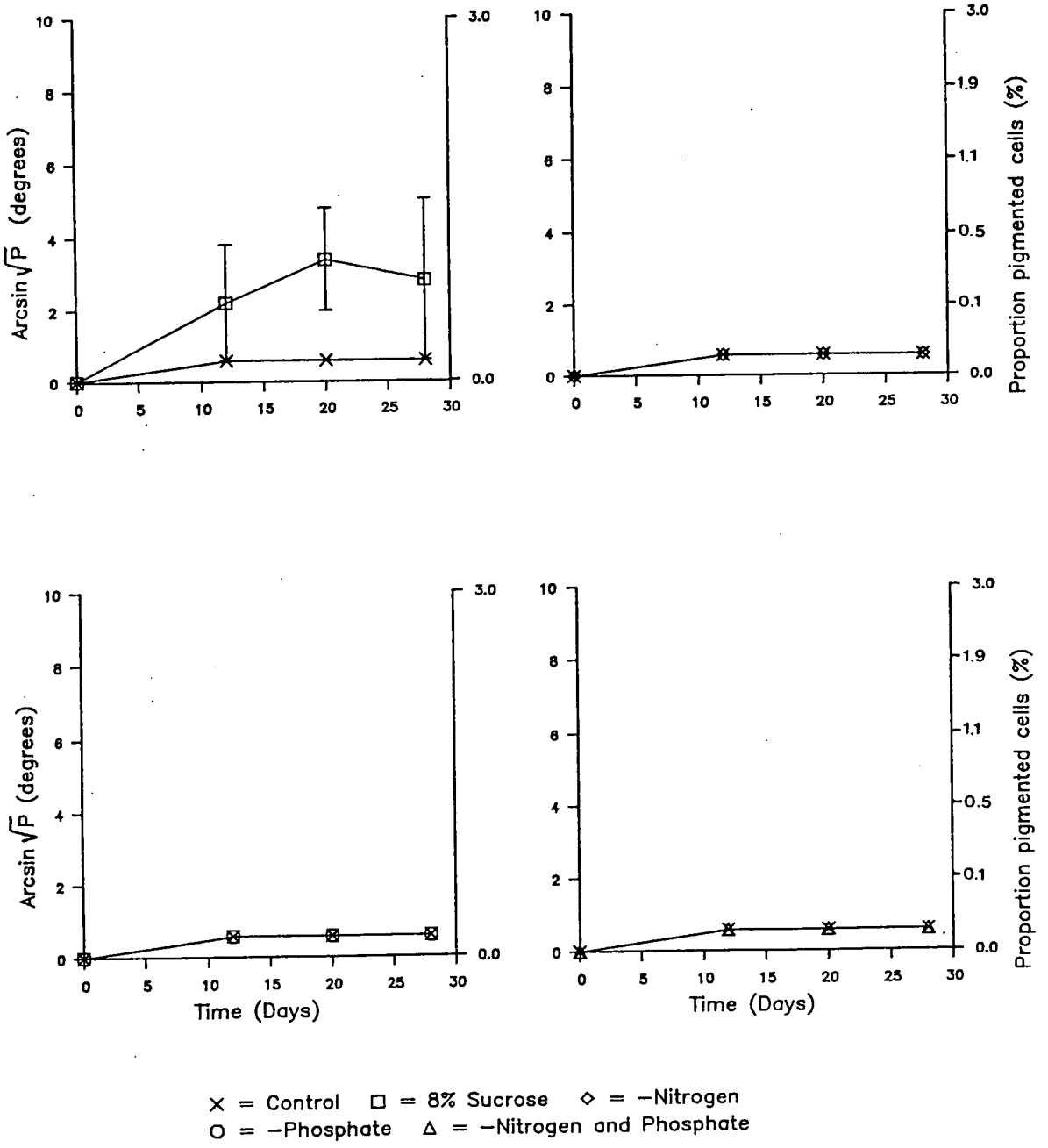
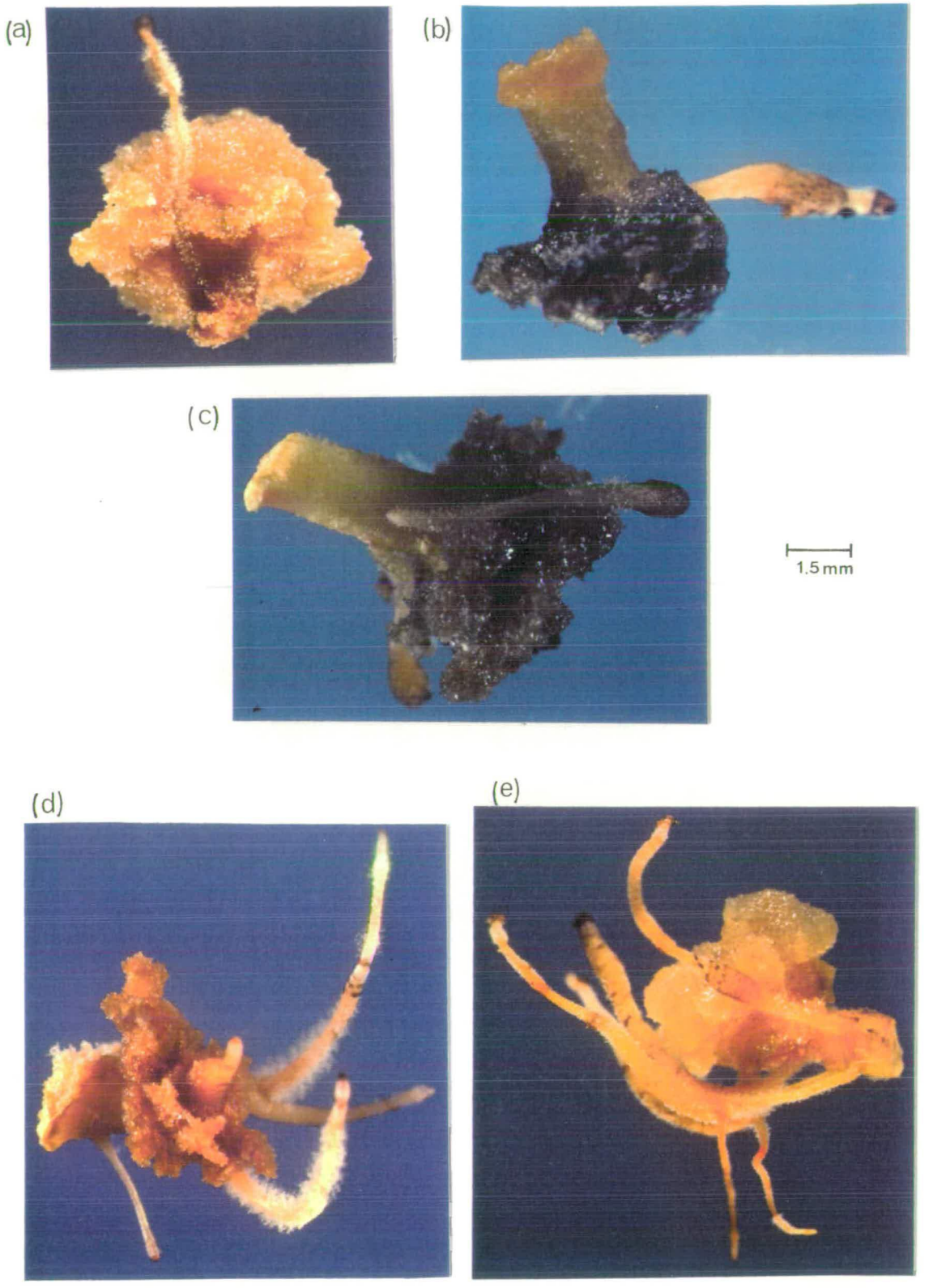


Figure 3.4.23

The appearance of root explants on (a) control, (b) MS-NP, (c) MS-P, (d) MS-N and (e) MS8%S media on d.28.



Summary of the Results in Section 3.4

The following points have emerged from the experiments detailed in this section.

(1) There was an increase in carotenoid pigment in the root and callus tissue during callus initiation from root tissue.

(2) This increase in pigment appears to be partly due to an increase in the number of pigmented cells.

(3) Altering the culture medium by adding 8% instead of 3% sucrose, or removing i-P and/or i-N resulted in a decrease in growth and an increase in pigment production during callus initiation from root tissue.

(4) The highest increase in pigment production occurred when the culture medium contained 8% sucrose.

(5) A significant increase in the proportion of pigmented cells was only obtained in the root tissue when the culture medium was lacking i-N.

Therefore, the results in this section show that both root and newly initiated callus tissue produced carotenoid pigments. When the culture medium was altered cell growth was reduced so primary metabolism was probably decreased. This could subsequently have led to a promotion of secondary metabolism which resulted in increased levels of carotenoid pigments. The highest increases in pigment occurred in both root and callus tissue when the medium contained 8% sucrose and an increase in the proportion of pigmented cells occurred in the root when the medium was lacking i-N. In the next section the carotenoid pigment levels during the batch growth of *B.orellana* suspension cultures were measured and the MS8%S and MS-N treatments were tested to investigate if pigment production could be increased.

3.5 INVESTIGATION OF PIGMENT LEVELS DURING THE BATCH GROWTH OF SUSPENSION CULTURES

In the last series of experiments it was shown that the amount of carotenoid pigment in newly initiated callus tissue could be increased by modifying the culture medium (see 3.4.). MS8%S, MS-N, MS-P and MS-NP treatments decreased the growth of root and callus tissue but they also increased the pigment levels compared to the control. In this section the pigment levels in suspension cultures were examined to determine whether pigment accumulation could be correlated with stage of development in the culture cycle. Also, the culture medium was altered in an attempt to increase pigment production in suspension cultures and the pigments were analysed using TLC and visible spectra analysis.

3.5.1 Measurement of Changes in Carotenoid Pigment Levels During the Batch Growth of Suspension Cultures

In the previous section (3.4) it was found that newly initiated callus tissue could produce carotenoid pigments. The aim of this experiment was to measure carotenoid pigment levels during the batch growth of suspension cultures.

The production of secondary metabolites by cell cultures has been shown to fluctuate during batch culture and to depend on factors such as the age and size of the inoculum. In many instances the production occurs during a particular phase of growth (King and Street, 1977). Evidence in the literature also suggests that an inverse relationship exists between culture growth and secondary metabolite accumulation (Yeoman *et al.*, 1980, 1982).

On day 0, several 14d. old cultures (100ml. flasks), grown on MS medium (see 2.2.1.2) were combined aseptically in a 500ml. conical flask. This was mixed thoroughly, filtered through a sieve (64μm.) and the filtered cells placed in several sterile 9cm. Petri dishes. 1g. of filtered cells was then added aseptically to 24 100ml. conical flasks containing 20ml. sterile MS medium. These flasks were placed on a rotary shaker under standard culture conditions (2.2.3.2). At regular time intervals three flasks were harvested at random for analysis. The contents of each flask were filtered and the fresh weight measured as described in 2.3.1.1. Two small representative samples of these cells (*ca.* 50mg.) were removed and the cell number and proportion of pigmented cells were determined as described in 2.3.1.3 and 2.3.3.4 respectively. The remaining cells were used to determine the amount of carotenoid pigment present (see 2.3.3.1 and 2.3.3.3). A typical spectrum obtained from cells in

this experiment is shown in Fig. 3.5.1. This shows that there is an absorption maximum at 460nm. This maximum is different to that found in the previous section (3.4) which suggests that at some stage during the development of cultures the pigment(s) must have changed. The nature of this change is not known. To compare the pigment in different samples a comparison of the absorption maxima at 460nm. was made. The results are presented in Figs. 3.5.2, 3.5.3 and 3.5.4. No pigment was found in the culture medium.

The results for fresh weight (Fig. 3.5.2) show a typical batch growth curve. There was a lag phase for the first three days when the cells were adapting to their environment and during that time no increase in fresh weight occurred. Between d.3 and d.21 the fresh weight increased 10.7 fold and then remained constant until the end of the culture period. This shows that the stationary phase, when growth had ceased probably due to lack of essential nutrients, was reached by d.21. This pattern of growth is similar to that seen previously in 3.2.2.3.

Changes in pigment levels in the cultures are shown in Fig. 3.5.3(a). Between d.0 and d.14 there was a 5.6 fold increase in the carotenoid pigment per flask. The pigment level then remained constant until d.28. The data presented in Fig. 3.5.3(b) show that the pigment per g. fresh weight increased 1.7 fold between d.0 and d.3 then decreased 3.2 times between d.3 and d.17. After d.17 the pigment per g. fresh weight remained constant. This shows that pigment production coincides with growth.

From the data presented in Fig. 3.5.4(a) it can be seen that there was no significant difference in the cell number per flask during the first three days. However, between d.3 and d.17 there was a 7.9 fold increase followed by a 1.3 fold decrease between d.17 and d.21. After d.21 the cell number per flask remained constant. The results for cell number per g. fresh weight (Fig. 3.5.4(b)) show that there was a 1.2 fold decrease between d.0 and d.3 followed by an increase (1.2 fold) between d.3 and d.7. The cell number per g. fresh weight then decreased 2 fold between d.7 and d.21 after which it remained constant. These results show that in the first three days the cells increased in size due to cell expansion. Then between d.3 and d.7 they became smaller as cell division began. Between d.7 and d.17 both cell expansion and cell division would have been occurring. Fig. 3.5.4(a) shows that cell division stopped after d.17 because there was no further increase in the number of cells per flask. However, Fig. 3.5.4(b) shows that the cells continued to expand between d.17 and d.21 since the cell number per g. fresh weight decreased. This is

also shown in Fig. 3.5.2 where the increase in fresh weight between d.17 and d.21 would have been due to cell expansion.

The results for the proportion of pigmented cells are not presented here as none were detected in the samples during the culture period. However, pigmented cells were occasionally observed in the cultures using an inverted microscope. This apparent inconsistency could be explained by the very small number of pigmented cells present in the culture.

Overall these results show that carotenoid pigment was present in the suspension cultures of *B.orellana* and this pigment increased during the culture period. Increases in pigment production occurred during the lag and early growth phases of batch growth. During the first three days there was an increase in pigment per g. fresh weight but a decrease in the cell number per g. fresh weight. Therefore, since no new cells were produced, either the existing cells were producing more pigment per cell or non-pigmented cells became pigmented. Between d.3 and d.14 the pigment per flask continued to increase. This increase could have been due to the reasons already stated or perhaps because some new cells produced by cell division were pigmented. Having found that suspension cultures of *B.orellana* produce carotenoid pigment attempts were made in the next experiment to increase the pigment level by manipulating the culture medium.

Figure 3.5.1

A typical spectrum obtained for the total carotenoid pigment extracted from 14d. old suspension cultures of *B.orellana*.

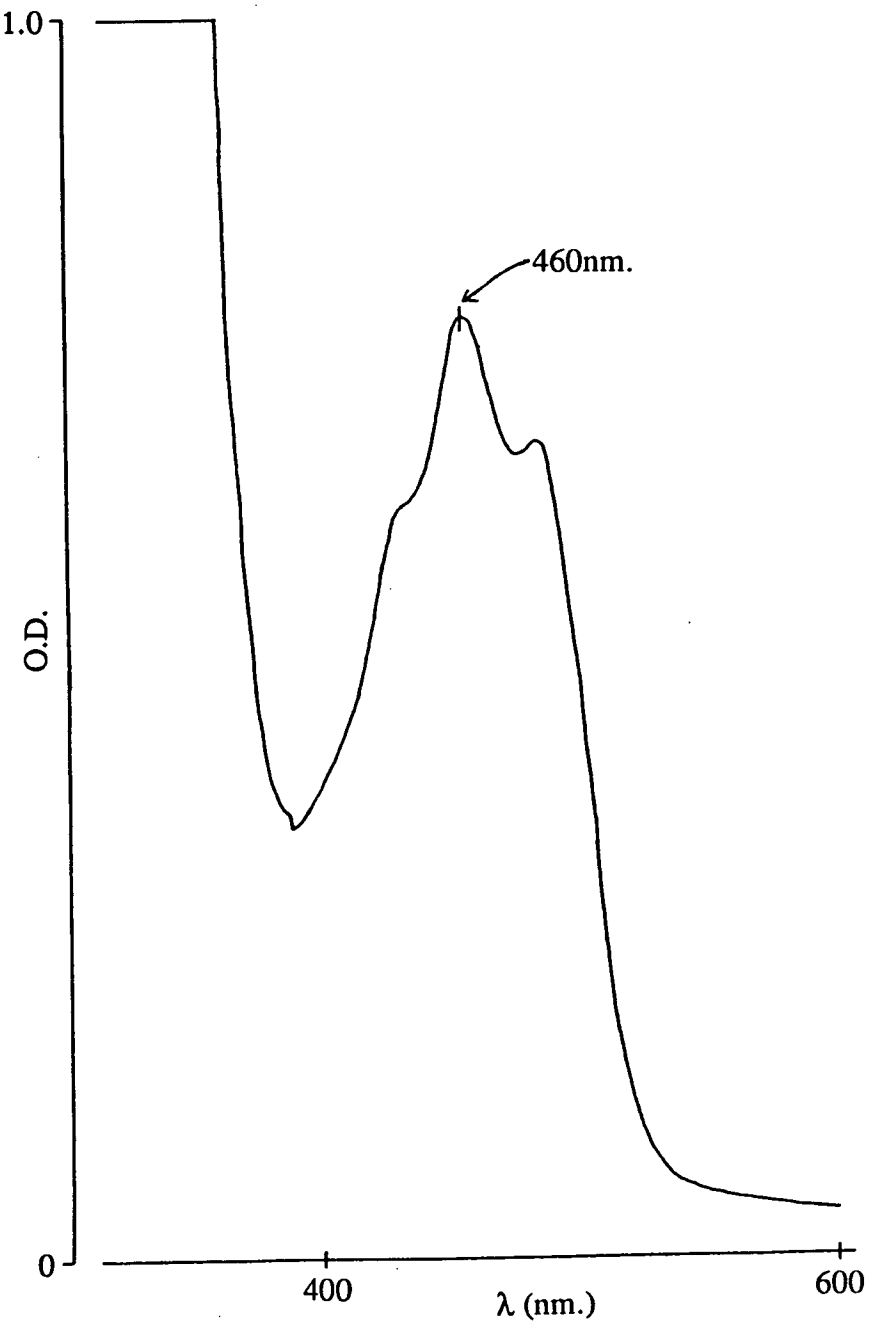


Figure 3.5.2

Changes in the fresh weight of suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

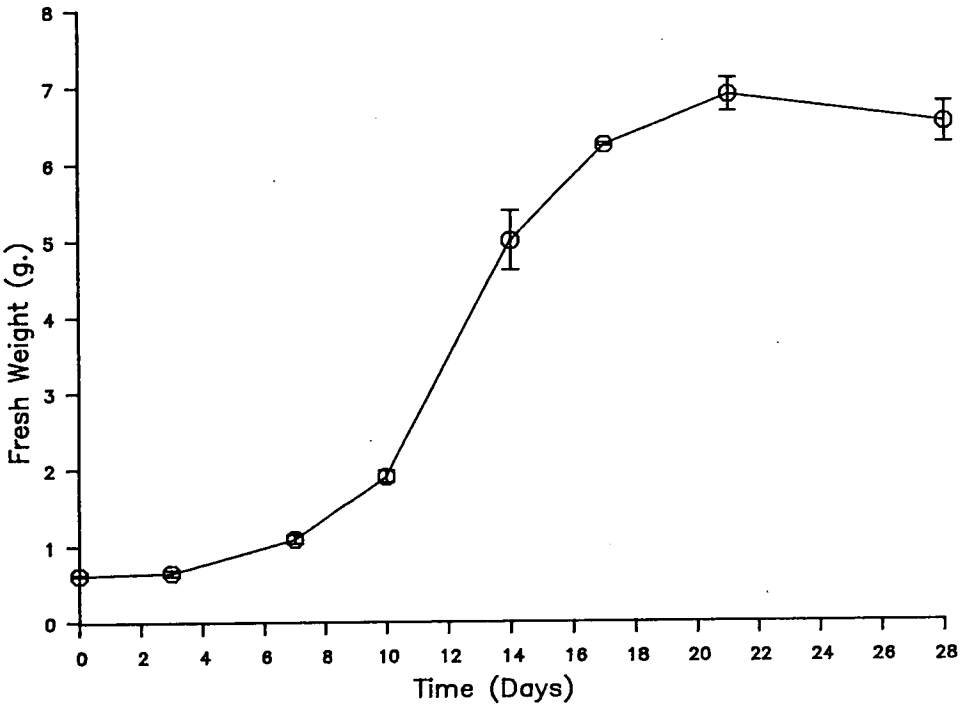


Figure 3.5.3

Changes in total carotenoid pigment per flask (a) and per g. fresh weight (b) measured at 460nm. in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

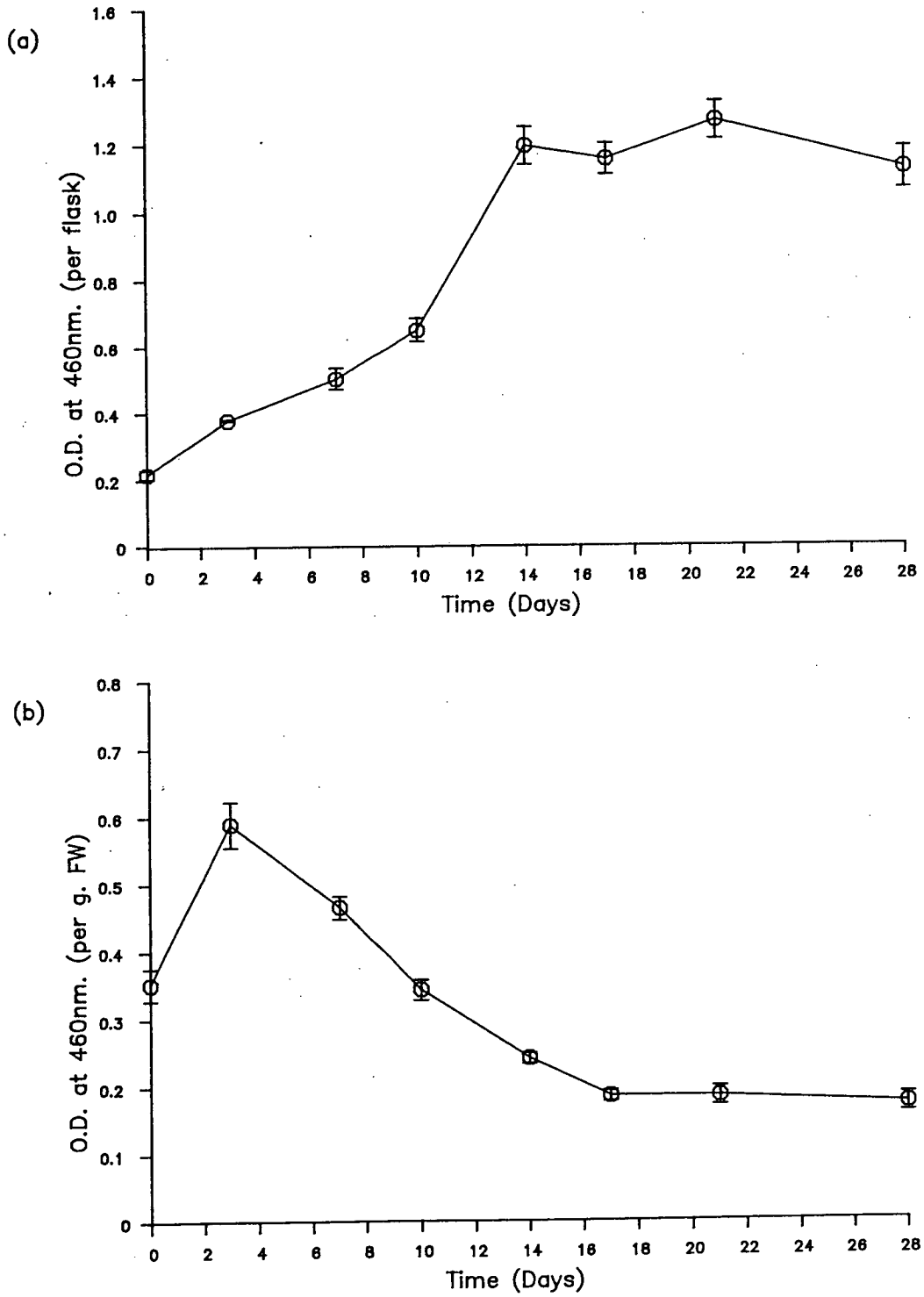
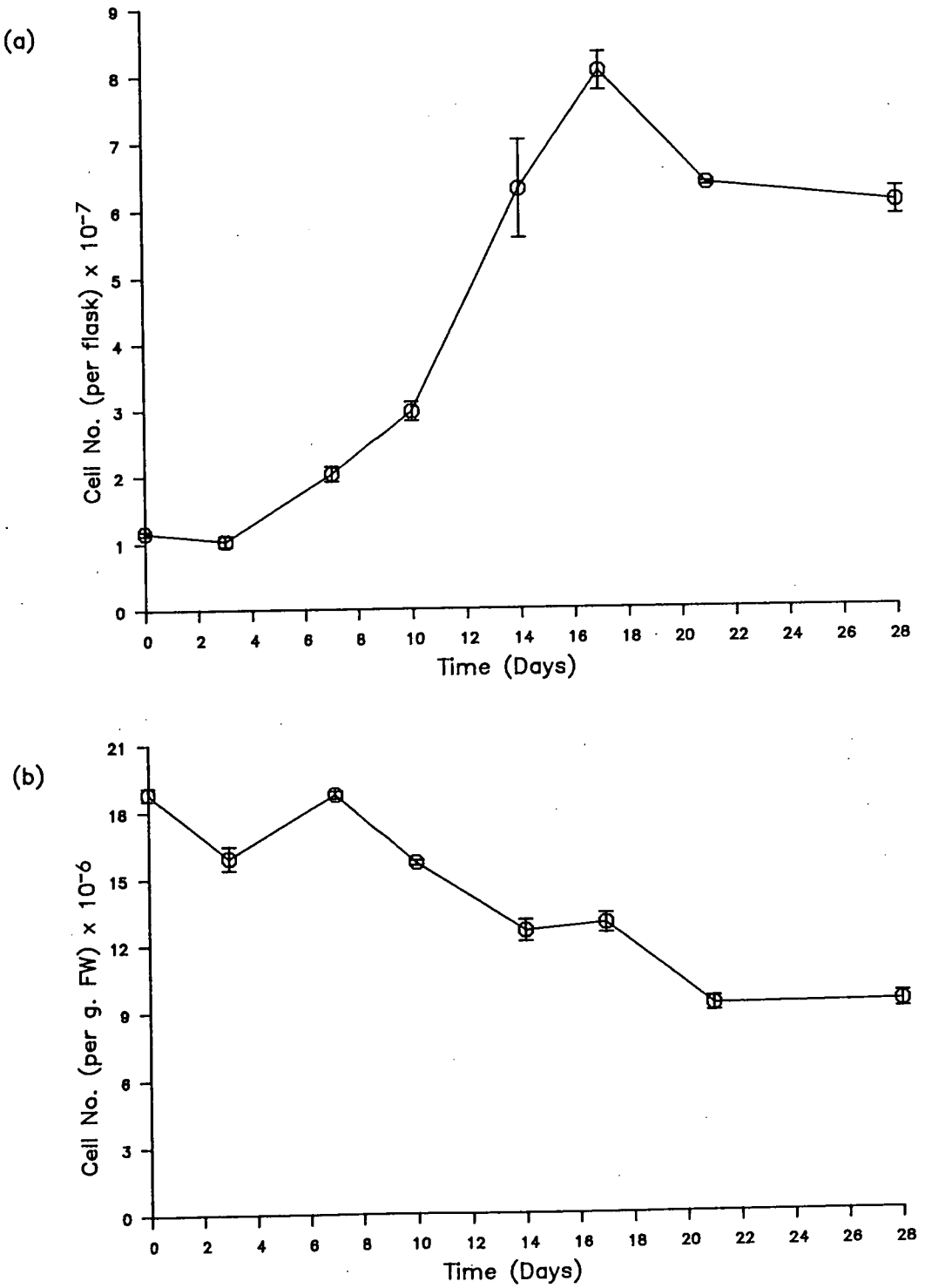


Figure 3.5.4

Changes in cell number per flask (a) and per g. fresh weight (b) in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.



3.5.2 Determination of the Effect of Nutrient Stress on Pigment Levels During the Batch Growth of Suspension Cultures

Previously it was shown that suspension cultures of *B.orellana* produced carotenoid pigments during batch growth (3.5.1). This production occurred predominantly in the lag phase and early stages of growth. Also in experiment 3.4.3 it was shown that pigment production in root and callus tissue increased when the culture medium was altered. The highest increases in pigment occurred in both root and callus tissue when the medium contained 8% sucrose and an increase in the proportion of pigmented cells occurred in the root when the medium was lacking i-N. The aim of this experiment was to investigate if pigment production in suspension cultures could be increased by altering the culture medium. Medium lacking i-N and/or medium with 8% sucrose were tested.

On day 0, 100ml. conical flasks containing 20ml. sterile medium were inoculated with 1g. of filtered 14d. old suspension culture cells as described in the previous experiment (3.5.1). Four different media were used: MS medium (control), MS lacking in i-N (MS-N), MS with 8% instead of 3% sucrose (MS8%S) and MS with 8% sucrose lacking in i-N (MS8%S-N). These media are described in 2.2.1.2 and 2.2.1.5. The flasks were placed on a rotary shaker under standard culture conditions (2.2.3.2). At regular intervals three flasks were harvested for each treatment. The fresh weight, cell number, proportion of pigmented cells and the carotenoid pigment were estimated for each treatment as described in 3.5.1. The results are presented in Figs. 3.5.5, 3.5.6, 3.5.7 and 3.5.8. The appearance of the cultures on the different media is shown in Fig. 3.5.9.

Once again, the results for the control show a typical batch growth curve (Fig. 3.5.5). The control fresh weight increased 10.4 fold between d.0 and d.28 which is similar to the previous experiment (3.5.1). After d.28 the fresh weight remained constant. The treatments depressed the fresh weight of the cultures compared to the control up to d.28. On MS8%S there was no increase in fresh weight between d.0 and d.14, so the lag phase was much longer than the control. The fresh weight on MS8%S then increased 9.2 fold between d.14 and d.40 which resulted in it being less than the control on d.10, d.14, d.17 and d.28 (all at P=0.001), but by d.40 it had reached the level of the control. On MS-N the fresh weight was less than the control on d.3 (significant at P=0.05), similar to the control on d.10 then was significantly less than the control for the remaining culture period (at P=0.01 on d.14 and d.17; P=0.001 on d.28 and d.40). The fresh weight of the cultures on MS8%S-N was

significantly lower than the control throughout the culture period (at P=0.01 on d.10; P=0.001 on d.3, d.14, d.17, d.28 and d.40).

The results presented in Fig. 3.5.6(a) show that for the control the carotenoid pigment per flask was constant over the first three days which was followed by a 2.7 fold increase between d.3 and d.10 then a 2.1 fold decrease between d.10 and d.17. After d.17 the pigment level remained constant. All of the treatments increased the pigment over the control. On MS8%S the amount of pigment was similar to the control until d.10. However, after d.10 the pigment on MS8%S continued to increase until d.28, resulting in a higher level than the control on d.14, d.17 and d.28 (significant at P=0.01 on d.17; P=0.001 on d.14 and d.28). However, between d.28 and d.40 the amount of pigment on MS8%S decreased so that it was not significantly different from the control on d.40. The pigment on MS-N increased between d.0 and d.10 and was significantly higher than the control on d.3 (at P=0.05) but significantly less on d.10 (at P=0.01). After d.10 it was not significantly different from the control. On MS8%S-N the pigment level was similar to the control on d.0 and d.3. Subsequently it increased between d.3 and d.14 then remained constant reaching a value less than the control on d.10 (at P=0.05) but higher on d.14, d.17, d.28 and d.40 (at P=0.1 on d.28; P=0.05 on d.17; P=0.01 on d.14 and d.40). The pigment increased 5.3 fold on MS8%S, 1.4 fold on MS-N and 2.1 fold on MS8%S-N compared to the control. Therefore, the largest increase in pigment production occurred when the medium contained 8% sucrose.

The pigment per g. fresh weight (Fig.3.5.6(b)) for the control was constant for the first ten days. Between d.10 and d.17 the pigment decreased 6.6 fold then it remained constant until the end of the culture period. Once again, all of the treatments increased the pigment per g. fresh weight compared to the control. On MS8%S the pigment was similar to the control for the first three days. However, after d.3 the pigment was higher than the control until d.40 when it was not significantly different (significantly higher at P= 0.01 on d.10; P=0.001 on d.14, d.17 and d.28). On MS-N the pigment was significantly higher than the control on d.3 (at P=0.05). The pigment then decreased between d.3 and d.10 and was significantly less than the control on d.10 (at P=0.1). After d.10 the decrease in pigment on MS-N was less than the control resulting in the pigment level on MS-N being higher than the control between d.14 and d.40 (significant at P=0.001 on d.14; P=0.05 on d.17 and d.28; P=0.01 on d.40). On MS8%S-N the pigment per g. fresh weight was significantly higher than the control throughout the culture period (at P=0.05 on d.3; P=0.01 on d.10; P=0.001 on d.14, d.17, d.28 and d.40). The pigment per g. fresh

weight increased 17.3 fold on MS8%S, 5 fold on MS-N and 7.3 fold on MS8%S-N compared to the control. Once again the largest increase occurred when the medium contained 8% sucrose.

The data presented in Fig. 3.5.7(a) show that for the control the cell number per flask increased 8.6 fold between d.0 and d.17 then remained constant between d.17 and d.40. All of the treatments up to d.28 resulted in a lower cell number per flask compared to the control. On MS8%S the cell number was the same as the control for the first three days. However, it was less than the control on d.10 (at P=0.01), d.14 (at P=0.001), d.17 (at P=0.01) and d.28 (at P=0.05). Both MS-N and MS8%S-N were significantly less than the control throughout the culture period (MS-N at P=0.1 on d.3; P=0.05 on d.10 and d.14; P=0.01 on d.17 and d.28; P=0.001 on d.40 : MS8%S-N at P=0.01 on d.3, d.10, d.14 and d.17; P=0.001 on d.28 and d.40). The cell number per flask was only significantly higher than the control on d.40 for MS8%S (at P=0.01).

Changes in the cell number per g. fresh weight (Fig 3.5.7(b)) show that for the control there was no significant change in the first three days then it increased 1.3 fold between d.3 and d.10. This was followed by a 1.7 fold decrease between d.10 and d.28 after which the cell number remained constant. All of the treatments were similar to the control on d.3. On d.10, MS8%S-N was similar to the control but MS-N was significantly less than the control (at P=0.01). MS8%S appeared to be greater than the control on d.10 but was not significant. The cell number per g. fresh weight was higher than the control on d.14 (at P=0.1), d.17 (at P=0.001), d.28 (at P=0.01) and d.40 (at P=0.05) for MS8%S. On d.14, d.17 and d.28 the cell number for MS8%S-N was similar to the control but it was higher than the control on d.40 (significant at P=0.05). MS-N was similar to the control on d.14 and d.28 but was significantly less on d.17 (at P=0.05) and higher on d.40 (at P=0.1). Therefore, 8% sucrose appears to decrease the cell size compared to the control during the culture period. Initially over the first ten days on MS8%S the cells became smaller even although there was no significant increase in cell number per flask and therefore, no cell division taking place. On MS8%S-N the cells were a similar size to the control throughout the culture period except on d.40 when they were smaller. The cell size on MS-N was similar to the control on d.3, d.14 and d.28, larger than the control on d.10 and d.17 and smaller than the control on d.40.

The proportion of pigmented cells was constant for the control throughout the culture period (Fig. 3.5.8). Both MS-N and MS8%S-N were not significantly

different from the control. However, on MS8%S the proportion of pigmented cells was significantly higher than the control on d.14 and d.17 (at P=0.01). These results show that for MS8%S the increase in pigment is at least partly due to an increase in the number of pigmented cells. However, there may also have been an increase in the amount of pigment per cell. For the other treatments the increase in pigment could have been due to either of these reasons. The zero values for the control, MS-N and MS8%S-N treatments may have been due to the proportion of pigmented cells being below the sensitivity of the method.

Overall these results show that, except for MS-N on d.3 and d.10 and MS8%S on d.40, all of the treatments decreased the growth of the cultures. However, the treatments also increased the pigment level during the culture period compared to the control. Pigment production occurs predominantly in the lag phase and early stages of growth. However, production stops at the downturn of growth and the pigment level then remains constant. The highest increases in pigment occurred on MS8%S medium. Therefore, it was decided to further investigate the effect of sucrose concentration on growth and pigment production by testing a range of sucrose concentrations.

Figure 3.5.5

Changes in the fresh weight of suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

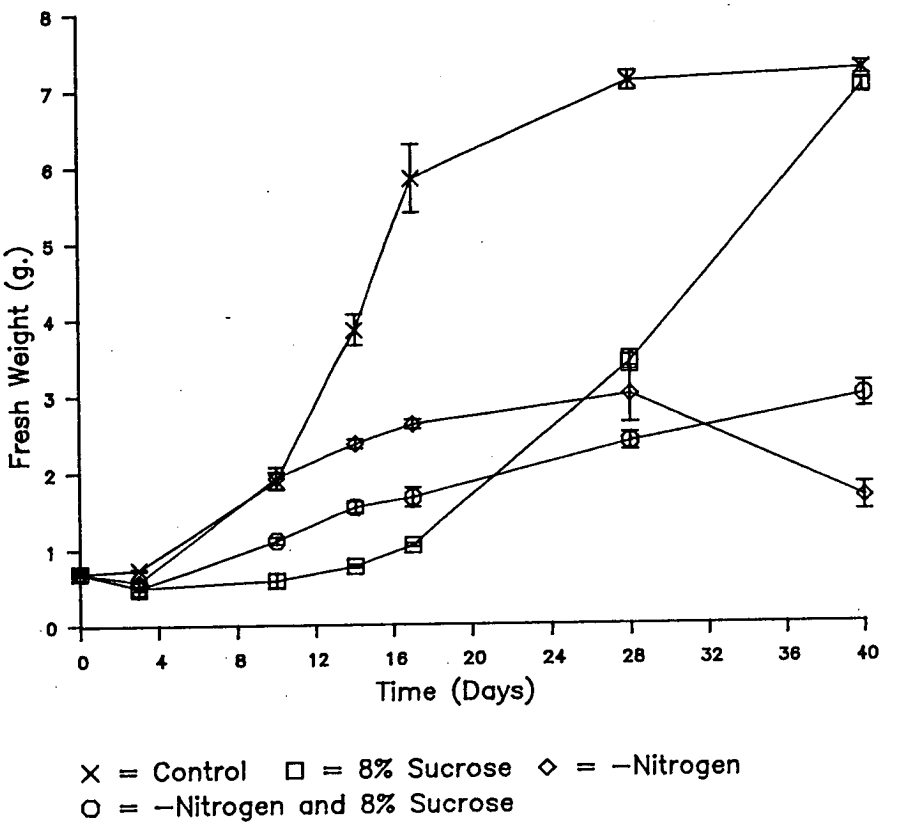


Figure 3.5.6

Changes in total carotenoid pigment per flask (a) and per g. fresh weight (b) measured at 460nm. in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

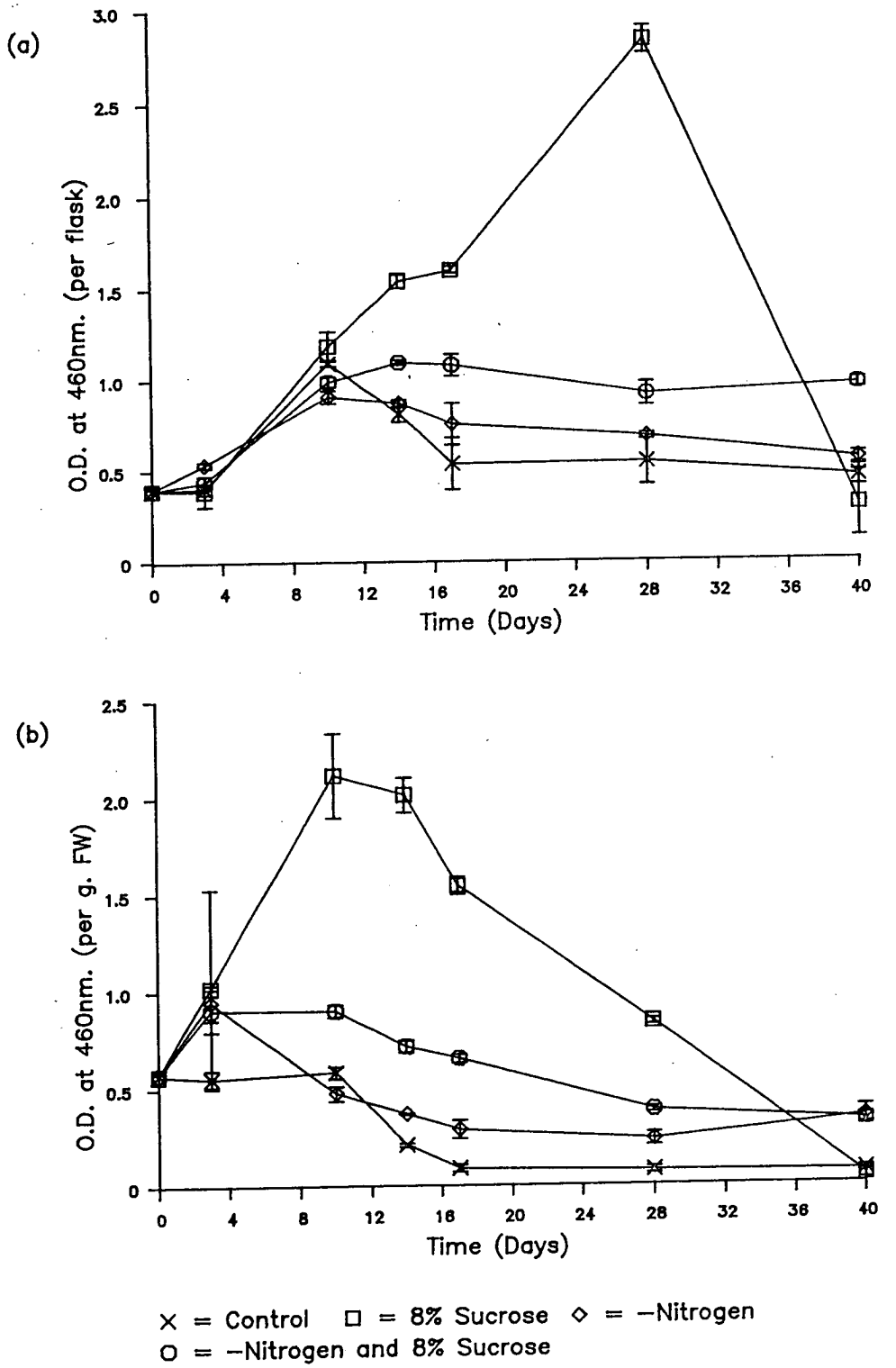


Figure 3.5.7

Changes in cell number per flask (a) and per g. fresh weight (b) in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

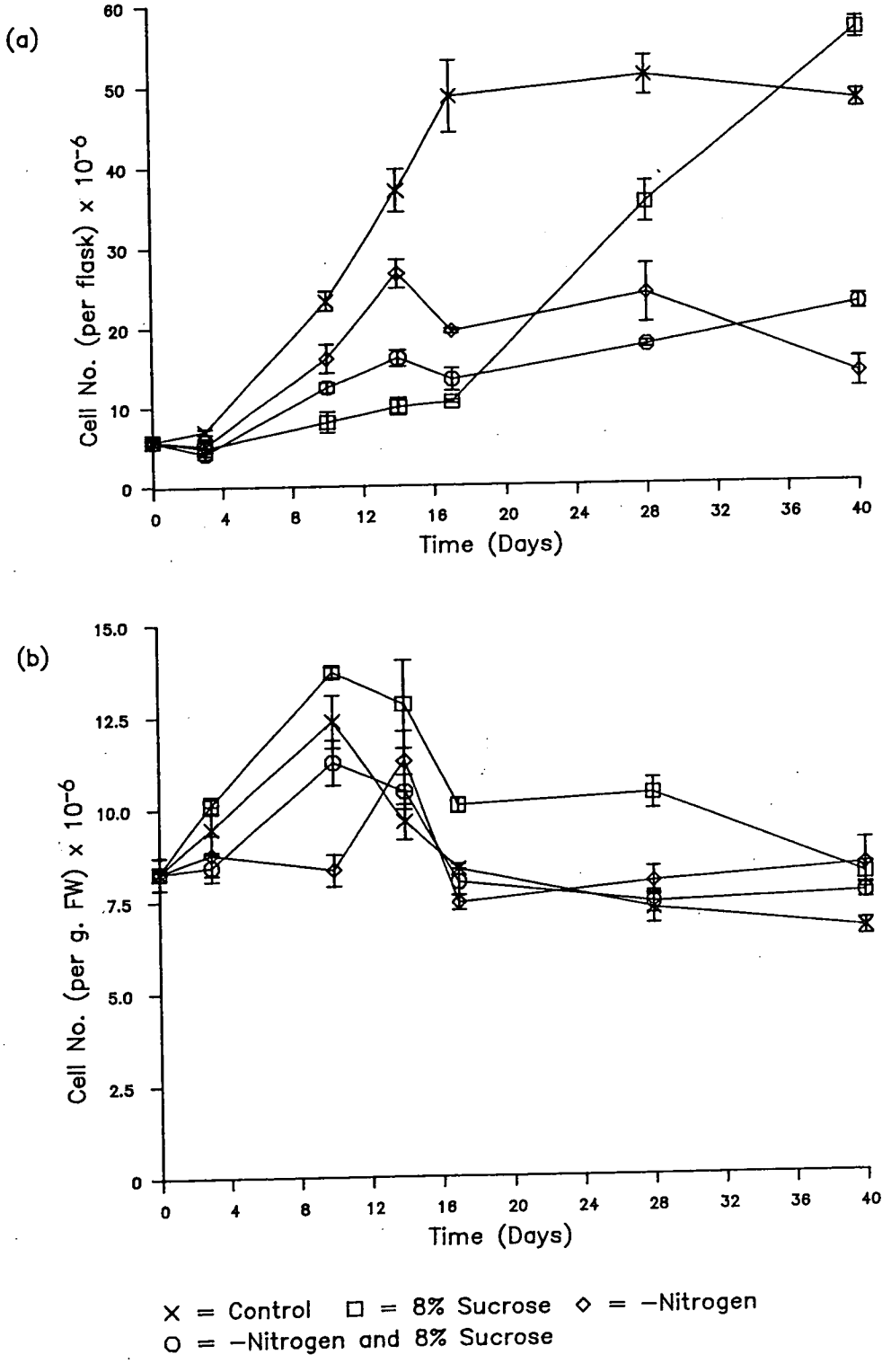


Figure 3.5.8

Changes in the proportion of pigmented cells in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

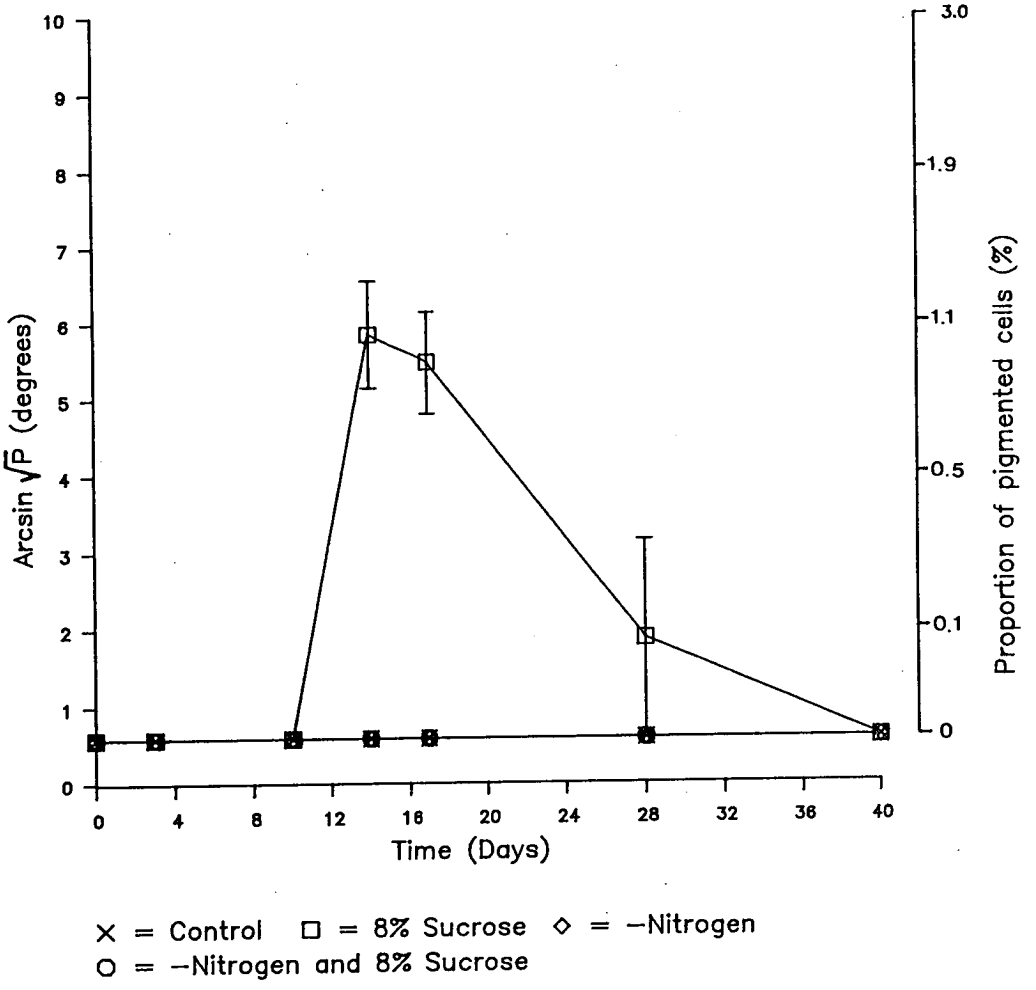
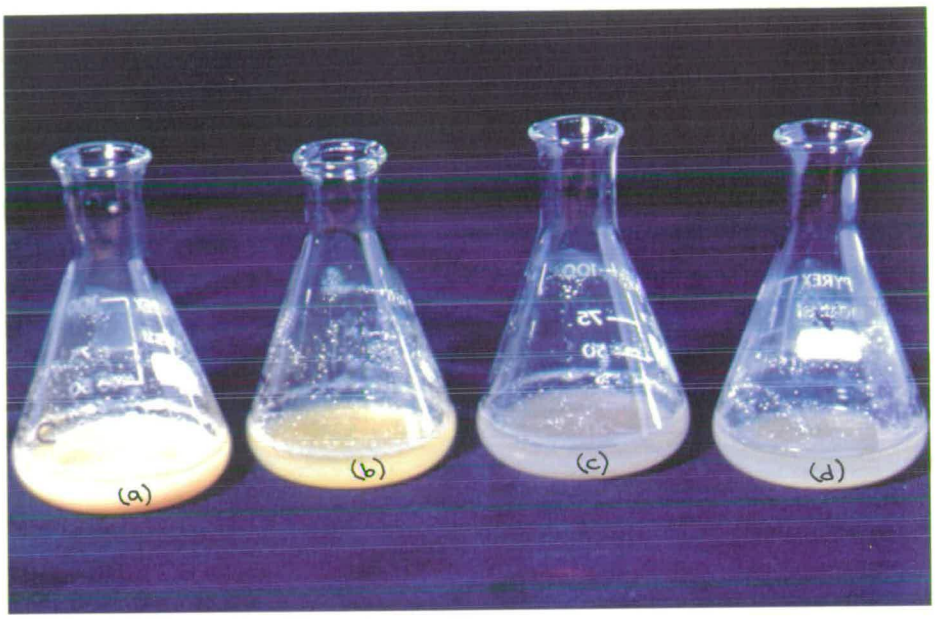


Figure 3.5.9

The appearance of suspension cultures grown on (a) control, (b) MS8%S, (c) MS-N and (d) MS8%S-N media on d.24.



3cm

3.5.3 Determination of the Effect of Sucrose Concentration on Pigment Levels During the Batch Growth of Suspension Cultures

In the last experiment (3.5.2) it was found that altering the major nutrients in the culture medium increased pigment production in suspension cultures of *B.orellana* during batch growth. The highest increase in pigment production occurred when the medium contained 8% sucrose. The aim of this experiment was to test a range of sucrose concentrations to investigate whether their effect on pigment production was related to the osmotic concentration of the sucrose or possibly due to the increased available carbon source.

As previously described 100ml. conical flasks were inoculated with 14d. old suspension culture cells and were maintained under standard culture conditions (see 3.5.1). In this experiment five different media were used: MS medium with 3% sucrose (control) (see 2.2.1.2), MS with 1% sucrose (MS1%S), MS with 6% sucrose (MS6%S), MS with 8% sucrose (MS8%S) and MS with 12% sucrose (MS12%S). On days 0, 3, 10, 17 and 24 three flasks were harvested for each treatment. The contents of each flask were filtered and the fresh weight, cell number, proportion of pigmented cells and the carotenoid pigment were estimated for each treatment as described in 3.5.1. The sucrose concentration and the osmolality of the medium were also determined at each time point as described in 2.3.2. The results are presented in Figs. 3.5.10, 3.5.11, 3.5.12, 3.5.13 and 3.5.14. The appearance of these cultures on d.24 is shown in Fig. 3.5.15.

The changes in the fresh weight of the controls (Fig. 3.5.10) are typical of a batch growth curve. There was a lag phase in the first three days followed by an 11.4 fold increase in fresh weight between d.3 and d.24. On MS1%S the fresh weight was similar to the control in the first three days then it increased more than the control between d.3 and d.10 resulting in the fresh weight being significantly higher than the control on d.10 (at P=0.001). After d.10 the fresh weight on MS1%S was constant so it was significantly less than the control on d.17 (at P=0.01) and d.24 (at P=0.001). The fresh weight for MS6%S was also similar to the control over the first three days then it increased between d.3 and d.24. The result was that the fresh weight on MS6%S was significantly less than the control on d.10 and d.17 (both at P=0.01) but similar to that on d.24. On MS8%S and MS12%S the cultures grew much less than the control, therefore the fresh weight was significantly less than the control throughout the culture period (MS8%S at P=0.05 and MS12%S at P=0.01 on d.3; both at P=0.001 on d.10, d17 and d.24). These results show that as the sucrose

concentration increased the growth decreased. On MS1%S growth stopped after d.10 probably because there was no sucrose left in the medium (see Fig. 3.5.14(a)). Therefore, it would appear as expected that there is a relationship between growth and sucrose concentration.

The results presented in Fig. 3.5.11(a) show that for the control the carotenoid pigment per flask increased 4.2 fold between d.0 and d.10. It remained constant between d.10 and d.17 then decreased 2 fold between d.17 and d.24. On MS1%S the pigment was significantly greater than the control on d.3 (at P=0.05) but was less on d.10, d.17 and d.24 (at P=0.05, 0.01 and 0.05 respectively). The pigment on MS6%S was also similar to the control on d.3 then it was significantly higher on d.10, d.17 and d.24 (at P=0.01, 0.01 and 0.1 respectively). Both MS8%S and MS12%S were less than the control on d.3 (at P=0.01 and P=0.1 respectively). On d.10 MS12%S was similar to the control whereas MS8%S was higher (at P=0.05). The pigment for MS8%S and MS12%S was higher than the control on d.17 and d.24 (all at P=0.001). These results show once again that pigment production occurred in the early stages of growth. As growth proceeded the pigment was constant then decreased. Larger amounts of pigment were produced at higher sucrose concentrations. The largest increment in pigment production occurred when the culture medium contained 8% sucrose which suggests that it was the optimum concentration of sucrose.

The data presented in Fig. 3.5.11(b) show that for the control the carotenoid pigment per g. fresh weight was constant over the first three days then it decreased 10.3 fold between d.3 and d.24. All of the treatments were similar to the control on d.3. However, on MS1%S the pigment was less than the control on d.10 and d.17 (both at P=0.05) and on MS6%S it was higher than the control on d.10 and d.17 (both at P=0.01). The pigment on d.24 was similar to the control for both MS1%S and MS6%S. On MS8%S and MS12%S the pigment was higher than the control on d.10 (at P=0.001), d.17 and d.24 (at P=0.01 for MS8%S and P=0.001 for MS12%S). Once again there appeared to be a relationship between sucrose concentration and pigment production. The largest increment in pigment per g. fresh weight was obtained when the medium contained 12% sucrose.

Changes in the cell number per flask (Fig. 3.5.12(a)) show that there was no change in the first three days for the control then it increased 6.8 fold between d.3 and d.17. After d.17 the control cell number per flask remained constant. Both MS1%S and MS6%S were similar to the control on d.3. The cell number on MS1%S was significantly higher than the control on d.10 (at P=0.001) but less on d.17 and d.24 (at

P=0.01 and 0.001 respectively). The decrease in cell number per flask after d.10 for MS1%S was probably due to the carbon source being exhausted (see Fig. 3.5.14(a)). On MS6%S the cell number was less than the control on d.10 and d.17 (at P=0.01 and 0.1 respectively) but was similar on d.24. MS8%S and MS12%S were both significantly less than the control throughout the culture period (at P=0.05 on d.3; P=0.001 on d.10 and d.24; P=0.01 and 0.001 respectively on d17). Therefore, increasing the sucrose concentration appears to decrease the cell number per flask.

The data presented in Fig. 3.5.12(b) show that for the control the cell number per g. fresh weight was constant over the first ten days then it declined 1.3 fold between d.10 and d.17 before remaining constant until the end of the culture period. All of the treatments increased the cell number per g. fresh weight compared to the control. It was significantly higher than the control on d.10 and d.24 (both at P=0.05) for MS1%S, on d.24 (at P=0.05) for MS6%S, on d.17 (at P=0.01) for MS8%S and on d.17 (at P=0.05) and d.24 (at P=0.01) for MS12%S. Therefore, compared to the control all of the treatments resulted in a lower cell size during the culture period.

The proportion of pigmented cells in the control once again remained constant throughout the culture period (Fig. 3.5.13). On MS1%S the proportion was similar to the control. However, MS6%S, MS8%S and MS12%S all increased the proportion of pigmented cells. The proportion of pigmented cells was higher than the control on d.10 and d.17 for MS6%S and on d.10, d.17 and d.24 for MS8%S but the proportion was only significantly higher than the control on d.10 (at P=0.01) for MS6%S and MS8%S and d.17 (at P=0.001) for MS8%S. For MS12%S the proportion of pigmented cells was significantly higher than the control on d.10, d.17 and d.24 (all at P=0.001). The average values for the proportion of pigmented cells on d.17 (3.9%) and d.24 (4.3%) for the MS12%S treatment were similar to the proportion obtained for the plant tissues (3.1.2). Therefore, at sucrose concentrations higher than the control an increase in the proportion of pigmented cells and thus an increment in the number of pigmented cells occurred on MS6%S, MS8%S and MS12%S. However, there may also have been a rise in pigment per cell. The proportion of pigmented cells was zero for both MS1%S and the control. Therefore, the nature of the increase in pigment in both these treatments could not be determined but it may have been due to a rise either in the number of pigmented cells or the pigment per cell or both. Possible reasons for these zero values were outlined previously (3.5.2).

The results presented in Fig. 3.5.14(a) show changes in the sucrose concentration of the media during the culture period. The d.0 values were lower than expected,

especially at the higher sucrose concentrations. This was found to be due to a dilution in the sucrose concentration brought about by the addition of cells to the medium. It can be seen (Fig. 3.5.14(a)) that the sucrose concentration was constant over the first three days for all of the media. The increase between d.0 and d.3 for MS12%S was not significant. For both MS1%S and the control the amount of sucrose declined after d.3 and reached *ca.* zero by d.10 for MS1%S and by d.24 for the control. The sucrose concentration for MS6%S decreased 1.9 fold between d.3 and d.10 then remained constant between d.10 and d.17 before decreasing 1.3 fold between d.17 and d.24. For MS8%S the sucrose dropped between d.3 and d.10 (1.4 fold) then remained constant. A fall also occurred between d.3 and d.10 (1.6 fold) for MS12%S but this was followed by a 1.3 fold increase between d.10 and d.24. The rise in sucrose concentration on MS12%S may have been due to cells plasmolysing in the high sucrose medium and releasing their contents into the medium.

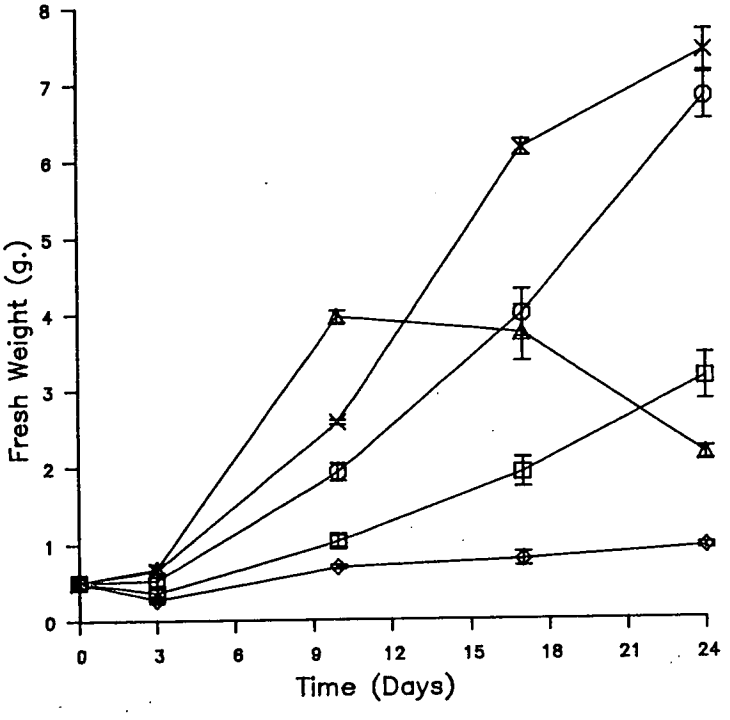
Changes in the osmolality of the medium are shown in Fig. 3.5.14(b). For MS1%S the osmolality increased 1.1 fold between d.0 and d.3 then decreased 2.4 fold between d.3 and d.10. Between d.10 and d.17 the osmolality remained constant but there was a further increment between d.17 and d.24 (1.2 fold). This rise towards the end of the culture period was probably due to cells dying and releasing their contents into the medium since there was a fall in the cell number per flask during that time (see Fig. 3.5.12(a)). The osmolality of the control increased 1.3 fold between d.0 and d.3 then decreased 15.4 fold between d.3 and d.24. For MS6%S and MS8%S there was a 1.3 fold rise in the osmolality of the medium in the first ten days. It then declined 1.8 fold between d.10 and d.24 for MS6%S but remained constant between d.10 and d.17 for MS8%S before decreasing 1.1 fold between d.17 and d.24. The osmolality increased 1.4 fold in the first 17d. then remained constant for MS12%S. It would appear from these results that at higher sucrose concentrations the osmolality increased initially for a longer period of time. This initial rise is probably due to the cells releasing metabolites as they equilibrate with the medium. Therefore, the longer increment at higher sucrose concentrations is probably due to the cells taking longer to equilibrate. The fall in osmolality appears to coincide with the start of growth (see Fig. 3.5.10).

From these results it would appear that increasing the sucrose concentration delays the growth of the cultures and therefore makes the lag phase longer. This may be due to the cells taking longer to adapt to their new medium. Cultures on MS1%S stopped growing after d.10 because there was very little sucrose left in the medium. Therefore, by d.24 the viability of these cultures was *ca.* 33%. The viability of the

cultures on the control, MS6%S, MS8%S and MS12%S was still high on d.24 (all *ca.* 79%). This shows that culture viability was not affected by high sucrose concentration. Once again, pigment production appeared to occur during the lag phase and early stages of growth. Therefore, more pigment was produced at higher sucrose concentrations because the growth was delayed. During the later stages of growth the pigment levels decreased to a very low level. On MS6%S, MS8%S and MS12%S an increase in the proportion of pigmented cells was observed and on MS6%S a decrease was also seen. These results suggest that the changes in pigment levels were at least partly due to changes in the number of pigmented cells. In the next experiment the nature of the pigments was determined by TLC and visible spectra analysis. Also a comparison was made between the pigments present in suspension cultures and those previously found in the plant tissues (see 3.1.3).

Figure 3.5.10

Changes in the fresh weight of suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.



△ = 1% Sucrose × = 3% Sucrose (control)
○ = 6% Sucrose □ = 8% Sucrose ◇ = 12% Sucrose

Figure 3.5.11

Changes in total carotenoid pigment per flask (a) and per g. fresh weight (b) measured at 460nm. in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

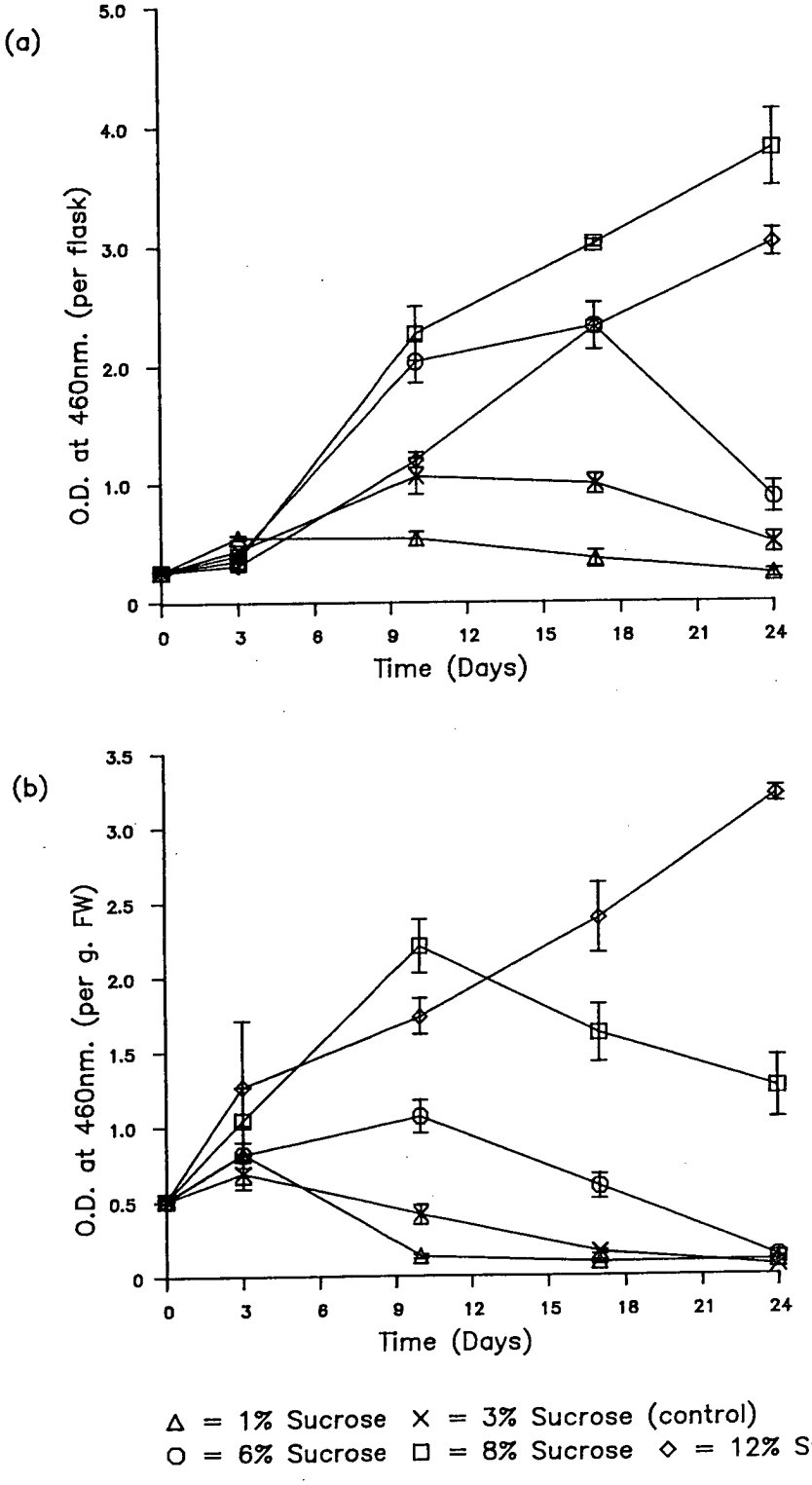


Figure 3.5.12

Changes in cell number per flask (a) and per g. fresh weight (b) in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

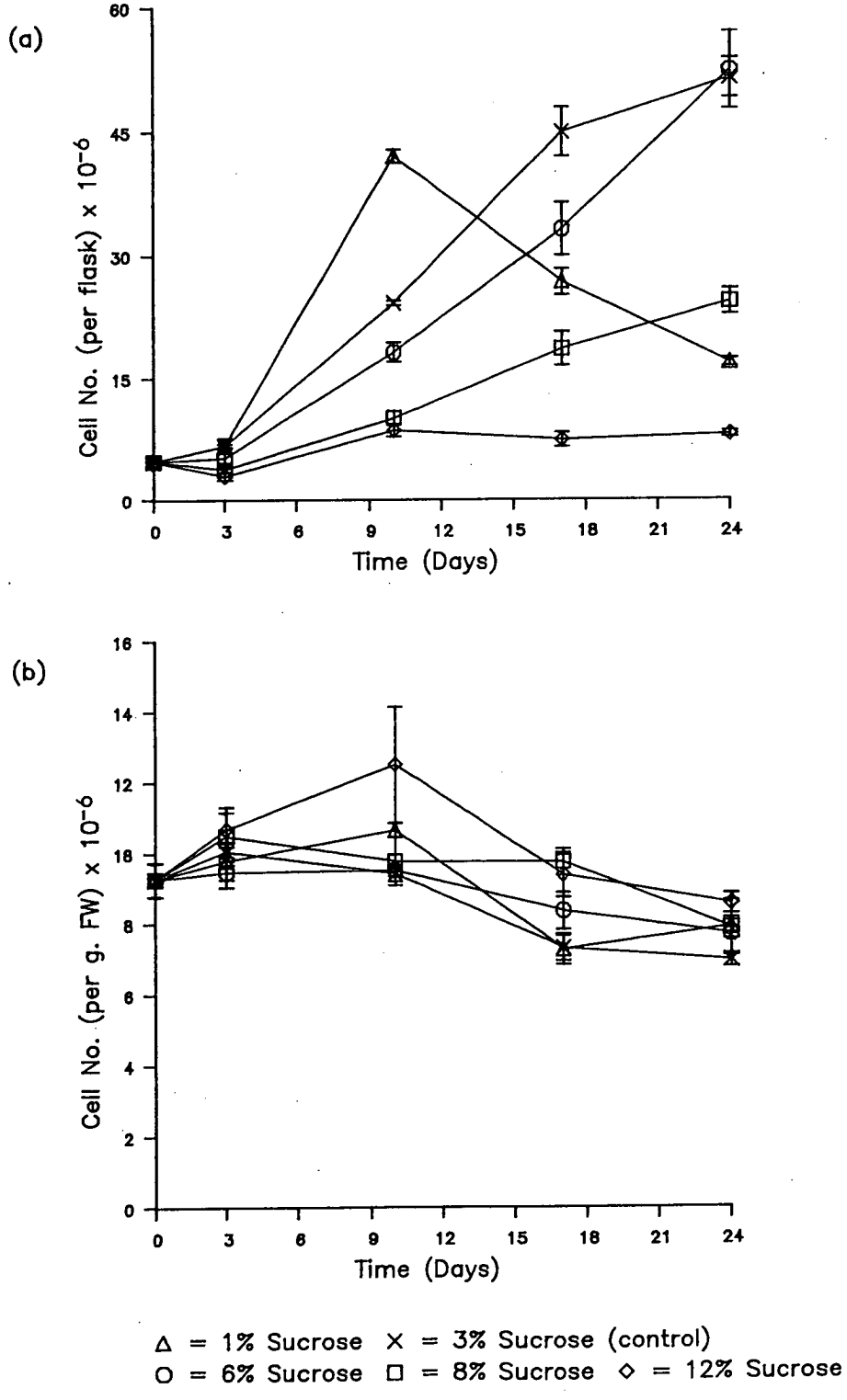


Figure 3.5.13

Changes in the proportion of pigmented cells in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

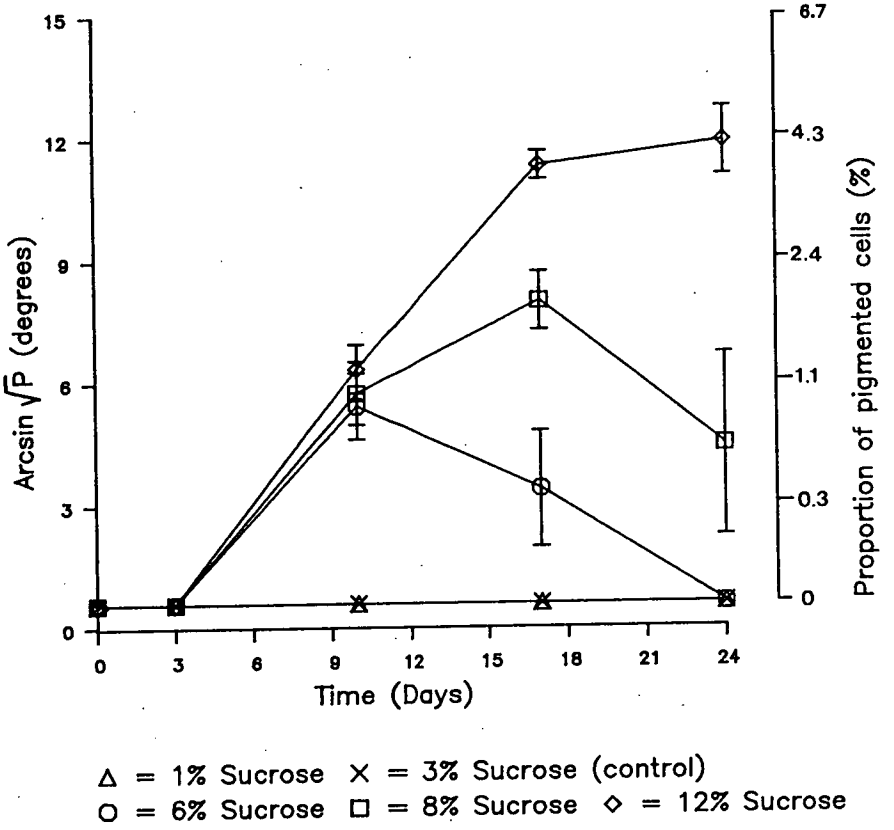


Figure 3.5.14

Changes in the sucrose concentration (a) and the osmolality (b) of the medium of suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

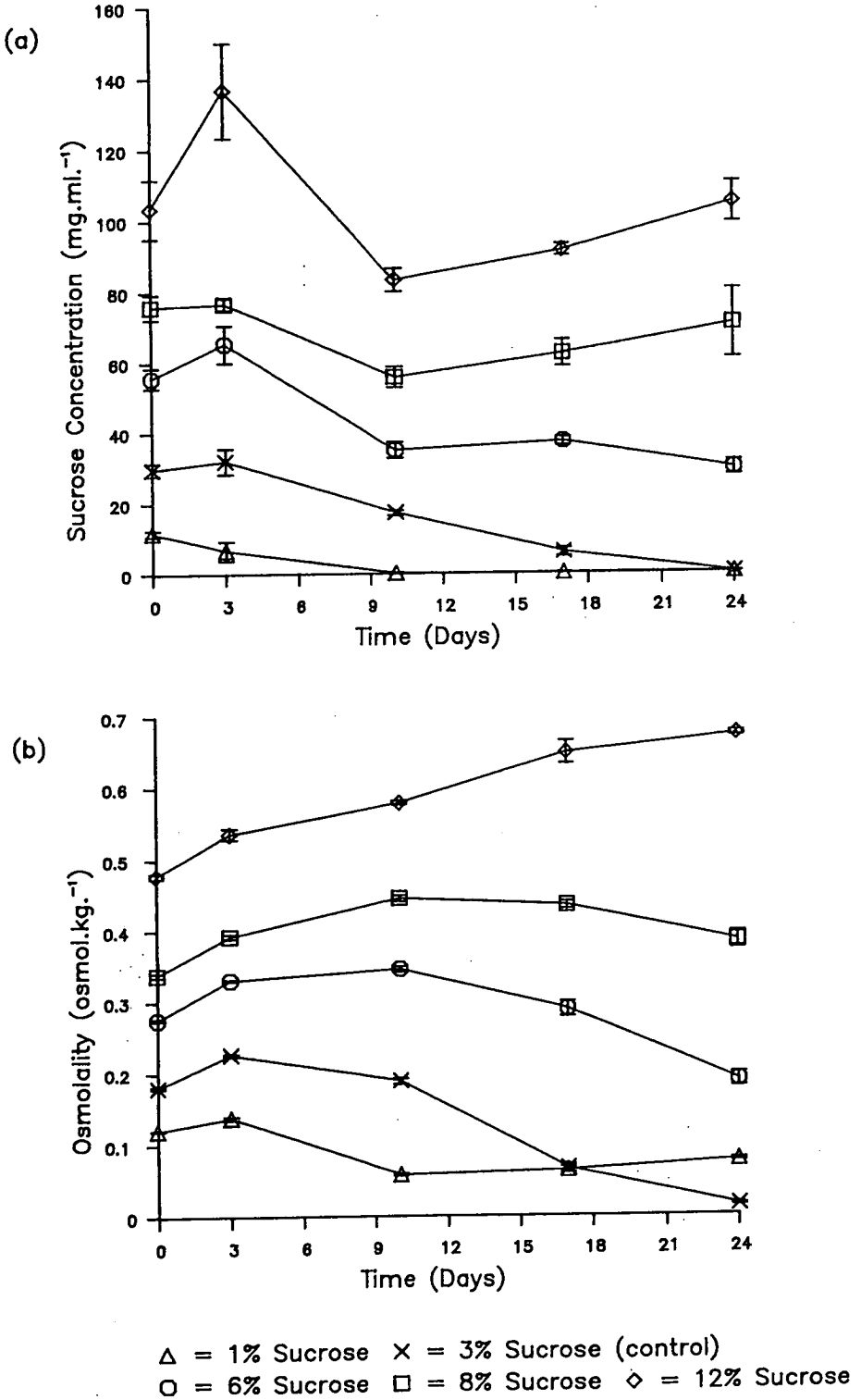
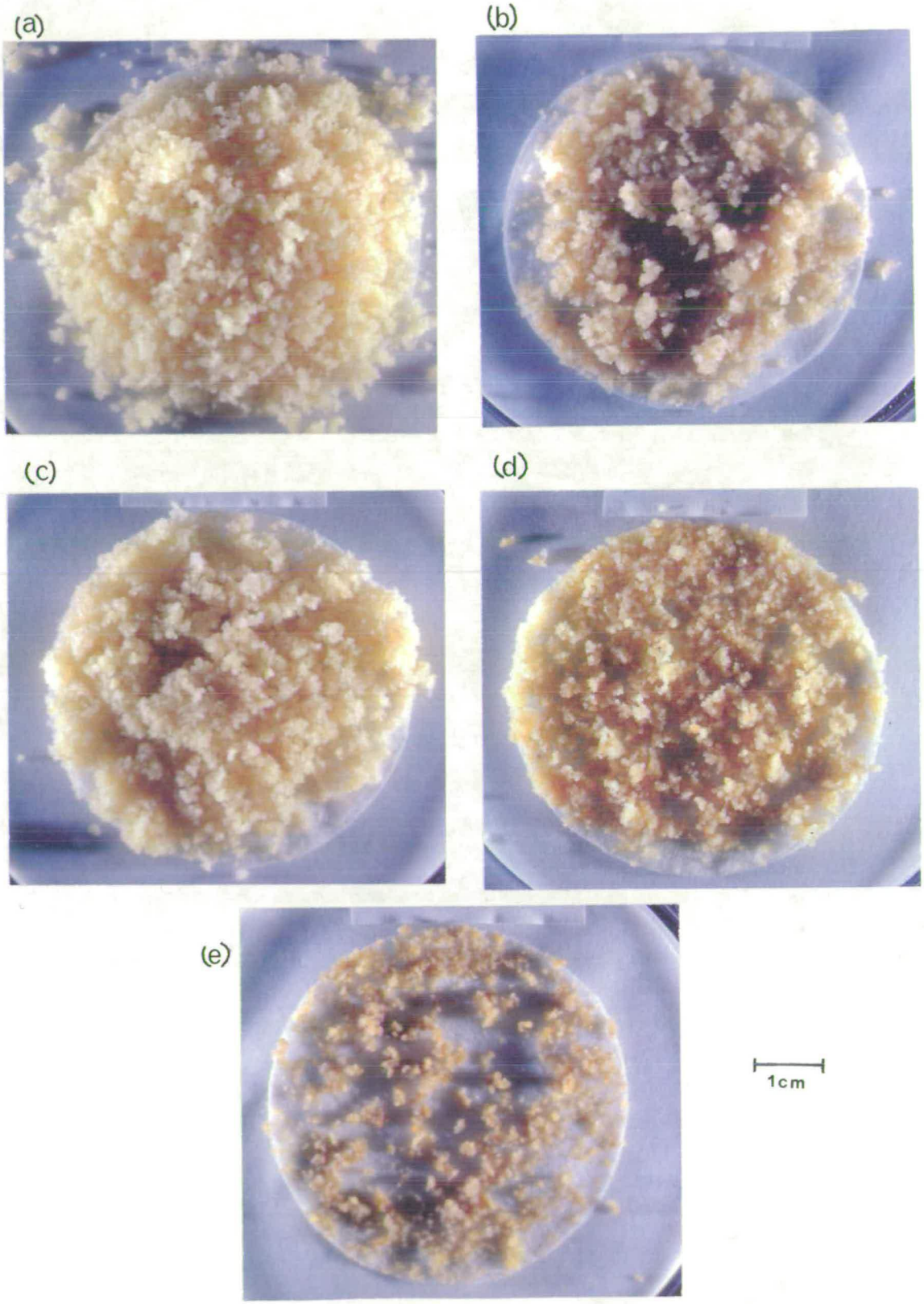


Figure 3.5.15

The appearance of suspension cultures grown on (a) control (3% sucrose), (b) MS1%S, (c) MS6%S, (d) MS8%S and (e) MS12%S media on d.24.



3.5.4 Analysis of Pigments in Suspension Cultures of *B.orellana*

In the previous experiment (3.5.3) it was shown that increasing the sucrose concentration in the medium resulted in a decrease in the growth of the cultures but it also increased the carotenoid pigment levels during the culture period. The aim of this experiment was to analyse the pigments present in suspension cultures grown on media with different sucrose concentrations using TLC and visible spectra analysis. The pigments present were also compared with those in the plant (3.1.3).

At time 0d., 100ml. conical flasks were inoculated with 1g. of 14d. old suspension cultures as described in 3.5.1. Two different media were used: MS medium with 3% sucrose (control) (see 2.2.1.2) and MS with 12% sucrose (MS12%S). The cultures were placed on a rotary shaker and maintained under standard culture conditions (2.2.3.2). On days 3 and 24 a flask was harvested for each treatment. The cultures were weighed then the carotenoid pigment was extracted as described in 2.3.1.1 and 2.3.3.1 respectively. The visible spectrum of each sample was measured (see 2.3.3.3) then the sample was loaded onto a reverse phase TLC plate (KC_{18}). Standards of bixin, lutein, zeaxanthin and β-carotene were also loaded onto the plate. The loaded plates were run for 45min. as described in 2.3.3.2 then the plates were dried and the pigment in orange/yellow coloured spots was obtained as described in 2.3.3.2. The samples of pigment obtained were dissolved in chloroform and the visible spectra were measured. Table 3.5.1 shows the O.D. for one of the λ_{max} values obtained for each of the pigments. The appearance of the TLC plates and the absorption spectra obtained are shown in Figs. 3.5.16 and 3.5.17 respectively.

The results show (Fig. 3.5.16) that three orange/yellow spots were obtained for the control on d.3 and d.24 and for MS12%S on d.3. However, on d.24 six spots were obtained for the MS12%S treatment. All of the spots had similar R_f values (see Fig. 3.5.17) to some of the spots obtained from the plant tissue (3.1.3) so the spots were numbered in a similar manner to those in 3.1.3. When spot 1 was analysed on d.3 and d.24 for both treatments there was not enough pigment present to obtain a visible spectrum so no absorption maxima were found. Spot 8 occurred on d.3 and d.24 for the control and MS12%S treatments. The R_f value and the spectrum obtained for spot 8 (Fig. 3.5.17) were similar to the zeaxanthin standard (see Fig. 2.3.2 for spectrum; $R_f = 0.38$). In the stem, leaf and petiole tissues spot 8 appeared to be lutein (see 3.1.3). Spot 10 was present on d.3 and d.24 for MS12%S and on d.3 for the control. From the R_f value and the spectrum obtained, spot 10 (Fig. 3.5.17) appeared

to be β-carotene (see Fig. 2.3.2 for spectrum of β-carotene; $R_f = 0.10$). Spots 6, 7 and 9 occurred only on d.24 for the MS12%S treatment. The R_f values and the spectra of these spots (Fig. 3.5.17) were similar to spots 6, 7 and 9 in the plant (3.1.3) but their identity is not known.

For the control the pigment levels in spots 8 and 10 (Table 3.5.1) decreased *ca.* 10 fold between d.3 and d.24 whereas for the MS12%S treatment the level of pigment in spots 8 and 10 increased. Between d.3 and d.24 there was also an increase in the number of pigments produced for the MS12%S treatment with the pigments corresponding to spots 6, 7 and 9 being produced. On d.3 the pigment levels (spots 8 and 10) for MS12%S were lower than the control but on d.24 the pigment levels were higher. Therefore, increasing the sucrose concentration in the medium leads to an increase in the pigment levels and an increase in the number of pigments present with time. There was no bixin present in the suspension cultures. However, with the exception of spot 8 (zeaxanthin) and possibly spot 1, the pigments in the suspension cultures were similar to pigments found in the plant (3.1.3). There was insufficient pigment present in spot 1 to be measured and determine if it was similar to the plant pigment. Less pigment was present in suspension cutures of *B.orellana* than in the plant tissues. There was *ca.* 1.2 (spot 10) to 900 fold (spot 6) more pigment in the plant than in suspension cultures.

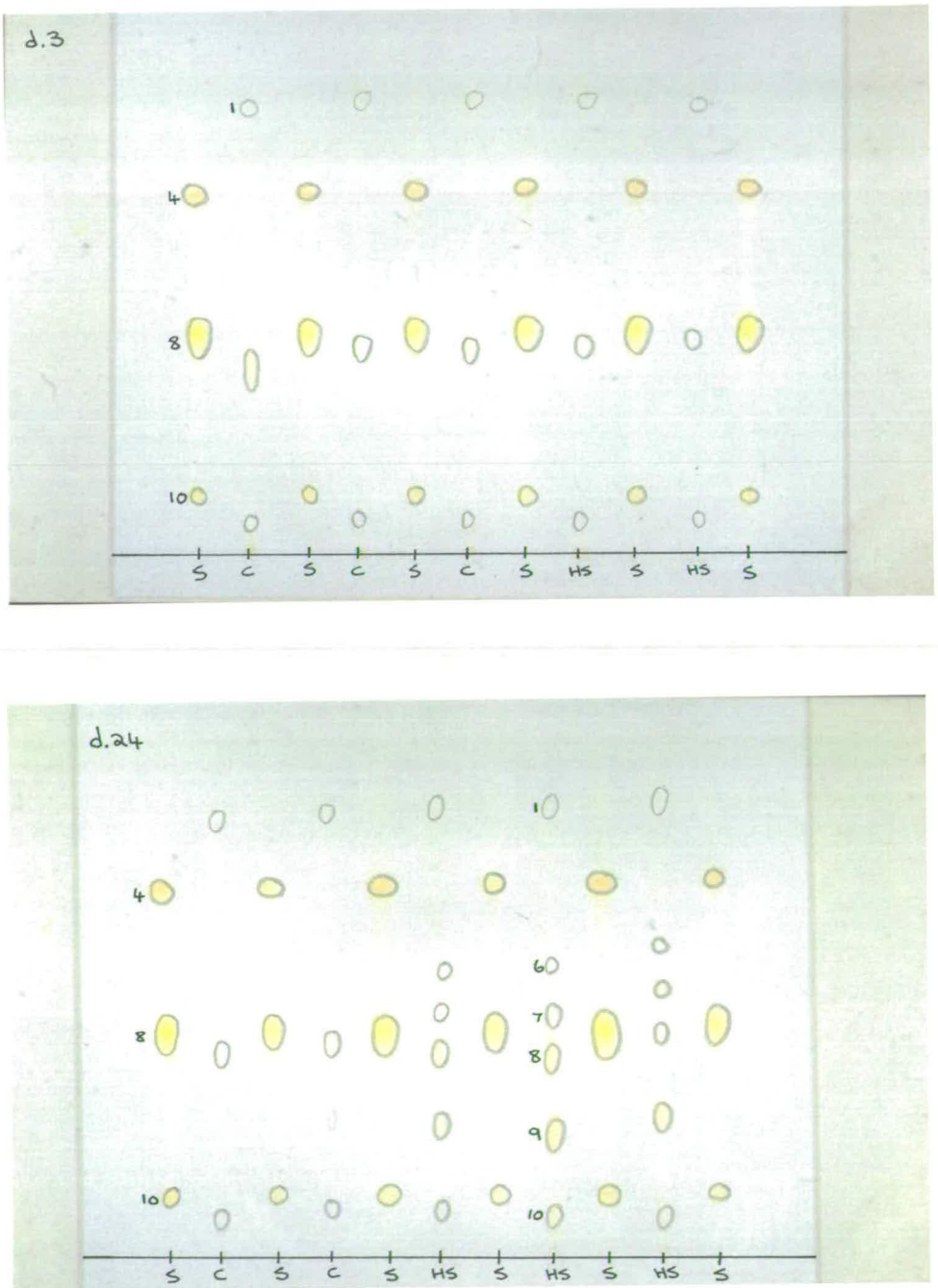
Table 3.5.1

O.D. values at a λ_{max} for each of the orange/yellow pigments obtained from suspension cultures of *B.orellana*.

Spot Number	λ_{max} nm.	O.D. at λ_{max} per g. fresh weight			
		Control		MS12%S	
		d.3	d.24	d.3	d.24
1	None	-	-	-	-
6	423-426	-	-	-	0.023
7	463-464	-	-	-	0.069
8	460	0.199	0.019	0.113	0.159
9	458-462	-	-	-	0.164
10	458-460	0.018	-	0.013	0.033

Figure 3.5.16

The appearance of TLC plates obtained from TLC analysis of suspension culture extracts of *B.orellana*.



S = standards (4 = bixin, 8 = zeaxanthin and lutein, 10 = β-carotene), C = control and HS = MS12%S

Figure 3.5.17

The appearance of visible absorption spectra obtained for spots 6, 7, 8 (a) and spots 9 and 10 (b) from TLC analysis of suspension culture extracts of *B.orellana*.

Figure 3.5.17(a)

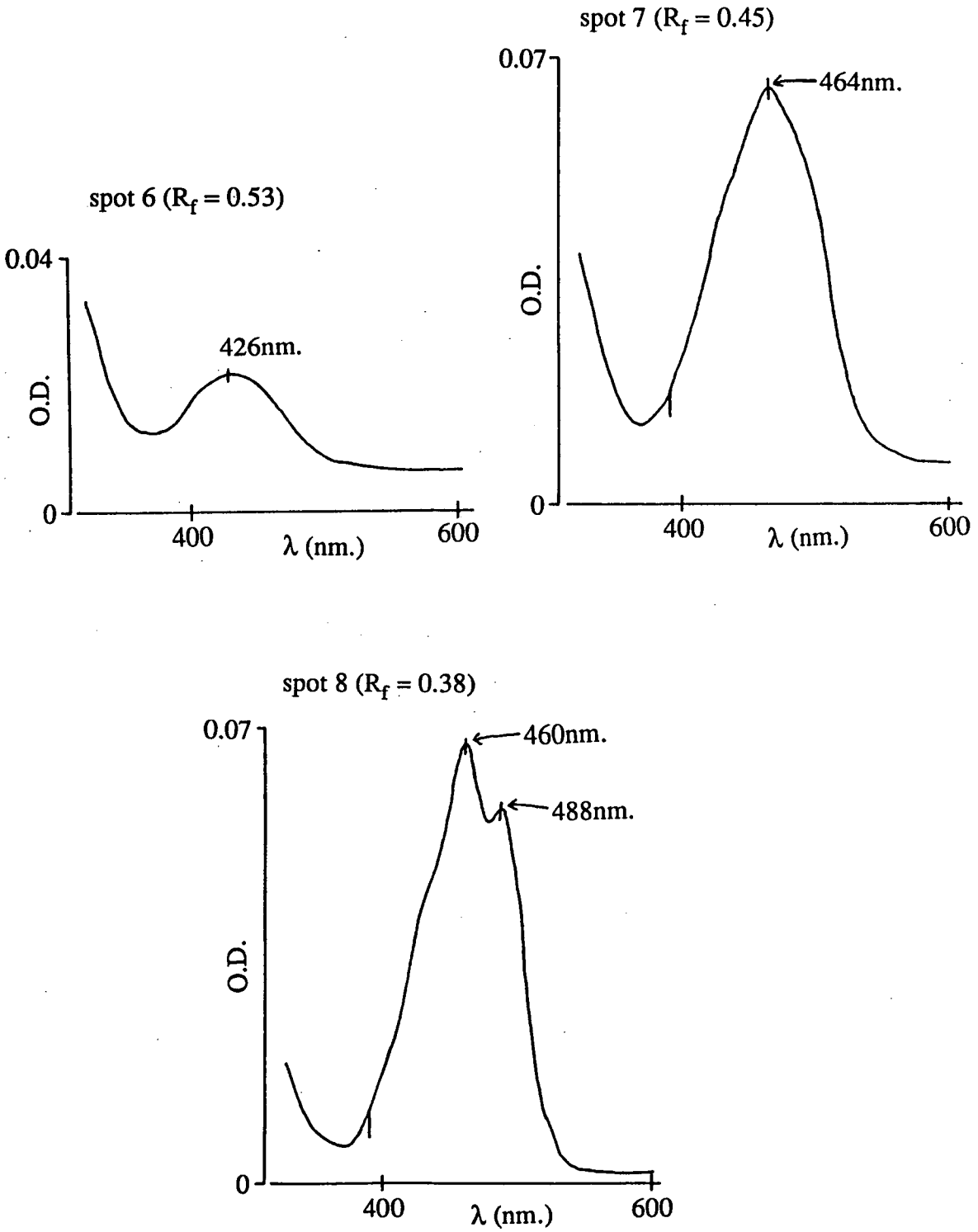
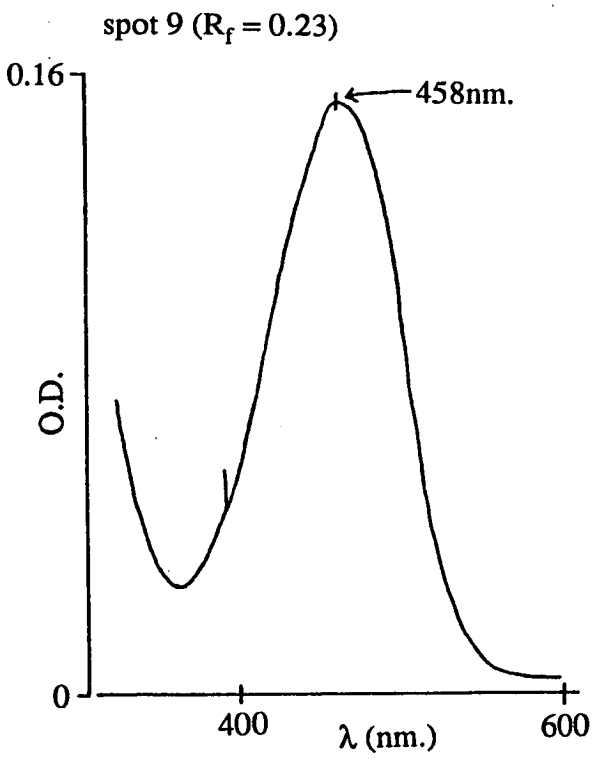
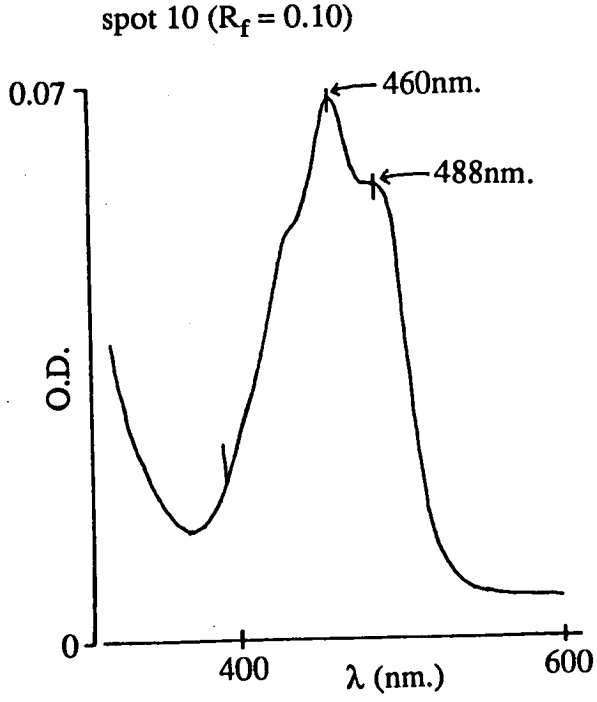


Figure 3.5.17(b)





Summary of the Results in Section 3.5

The following notable points have emerged from the work reported in this section.

(1) Carotenoid pigment was present in suspension cultures of *B.orellana* and this pigment increased during the culture period.

(2) During batch growth increases in pigment production occurred in the lag phase and early stages of growth.

(3) When the culture medium was manipulated (addition of 8% sucrose or removal of i-N) the growth of the suspension cultures decreased and the pigment levels increased during the culture period compared to the control.

(4) When the sucrose concentration in the medium was increased (6%, 8% and 12% instead of 3%) the growth of the cultures was delayed. Since pigment production occurred during the lag phase and early stages of growth, the delayed growth with 6%, 8% and 12% sucrose concentrations resulted in a rise in pigment production.

(5) The changes in pigment levels in suspension cultures were at least partly due to changes in the number of pigmented cells.

(6) Analysis of the pigments produced by suspension cultures on the control and MS12%S media showed that zeaxanthin and β-carotene were present. There was an increase in the amounts of these pigments and three more pigments of unknown identity were produced for the MS12%S treatment with time.

Overall the results show that carotenoid pigments were present in suspension cultures of *B.orellana*. Also the increased levels of pigment produced when the sucrose concentration in the culture medium was raised was shown to be due to an increase in the amount of zeaxanthin and β-carotene and the number of pigments present. However, from these results it was not determined whether the rise in pigment levels obtained at higher sucrose levels than 3% were due to an increase in the amount of available carbon or due to an osmotic effect. Therefore, in the next section the nature of this sucrose effect was further investigated to determine if the larger increases in pigment levels at higher sucrose concentrations were due to the increased osmolality of the medium.

3.6 AN INVESTIGATION TO DETERMINE WHETHER THE EFFECT OF SUCROSE CONCENTRATION ON PIGMENT PRODUCTION IS OSMOTIC

In the last series of experiments (3.5) it was found that increasing the sucrose concentration in the medium of suspension cultures of *B.orellana* was accompanied by a decrease in growth and an increase in the levels of various carotenoid pigments. Analysis of the pigments showed that there was an increase in the amount of zeaxanthin and β-carotene and an increase in the number of pigments present when the sucrose concentration was increased. The aim of the experiment described here was to establish whether these effects were due to the high osmotic concentration of sucrose in the medium. To test for an osmotic effect the osmolality of the control medium was adjusted by supplementing it with mannitol. Mannitol is widely used as an osmoregulator and this is based on the assumption that it either does not enter living cells, or if it does, it is not metabolized. Indeed, it has been reported that mannitol is not metabolized by tobacco (Trip *et al.*, 1964; Brown *et al.*, 1979).

On day 0, 100ml. conical flasks were inoculated with 14d. old suspension culture cells grown on MS medium as described in 3.5.1. Three different media were tested: MS medium with 3% sucrose (control) (see 2.2.1.2), MS with 12% sucrose (MS12%S) and MS with 3% sucrose and 51.5g.l.$^{-1}$ mannitol (MS3%S+M). Previously it was determined that this amount of mannitol when combined with 3% sucrose gave a solution with the same osmolality as 12% sucrose, but with the same available carbon as the control. The flasks were maintained under standard culture conditions (2.2.3.2) and on days 0, 3 and 24 three flasks were harvested at random for each treatment. The fresh weight, cell number, proportion of pigmented cells and the carotenoid pigment were estimated at each time point for the three treatments as described in 3.5.1. Both the osmolality and sucrose concentration of the media were also determined (see 3.5.3). The results are presented in Tables 3.6.1, 3.6.2, 3.6.3, 3.6.4 and 3.6.5. The appearance of the cultures on d.24 is shown in Fig. 3.6.1.

The results for the control fresh weight (Table 3.6.1) show that there was a lag phase for the first three days followed by a 14.1 fold increase between d.3 and d.24. This is similar to the pattern of growth seen previously in 3.5. For both MS12%S and MS3%S+M the fresh weight was similar to the control on d.0 and d.3. However, they were both significantly less than the control (at P=0.001) on d.24. The MS3%S+M treatment increased more than MS12%S between d.3 and d.24 so it was significantly higher than MS12%S on d.24 (at P=0.1). From these results it would appear that increasing the osmolality of the medium resulted in a decrease in the growth of the

cultures.

Changes in the carotenoid pigment per flask (Table 3.6.2(a)) for the control show that it increased 6 fold between d.0 and d.24. In both the MS12%S and MS3%S+M treatments the pigment levels were constant between d.0 and d.3 and so were significantly less than the control on d.3 (at P=0.01 and 0.05 respectively). However, the amount of pigment increased between d.3 and d.24 for both treatments such that MS3%S+M was similar to the control on d.24 but MS12%S was significantly higher than the control (at P=0.05) and MS3%S+M (at P=0.01) on d.24. It would appear from these results that the osmolality of the medium was not having an effect on pigment production per flask since the control was similar to MS3%S+M on d.24.

The data in Table 3.6.2(b) show that the carotenoid pigment per g. fresh weight in the control increased 1.4 fold over the first three days then decreased 3.9 fold between d.3 and d.24. MS3%S+M was similar to the control on d.3 but was significantly higher on d.24 (at P=0.05). The MS12%S treatment was also similar to the control on d.3 then it increased between d.3 and d.24 resulting in it being significantly higher than both the control (at P=0.001) and MS3%S+M (at P=0.05) on d.24. Therefore, increasing the osmolality of the medium results in an increase in the pigment per g. fresh weight.

The cell number per flask in the control (Table 3.6.3(a)) was constant over the first three days then increased 8.2 fold between d.3 and d.24. MS12%S and MS3%S+M were both similar to the control between d.0 and d.3, however, they were significantly less than the control on d.24 (both at P=0.01). The MS3%S+M treatment appeared to be higher than MS12%S on d.24 but was not significant.

Table 3.6.3(b) shows that the cell number per g. fresh weight for the control increased 1.1 fold between d.0 and d.3 then decreased 1.7 fold between d.3 and d.24. MS12%S was similar to the control on d.3 but was higher on d.24 (significant at P=0.05). The MS3%S+M treatment was significantly higher than the control on d.3 (at P=0.05) and d.24 (at P=0.05), however, it was not significantly different from MS12%S. Therefore, increasing the osmolality of the medium leads to a decrease in cell size since there were more cells per g. fresh weight for MS12%S and MS3%S+M.

The proportion of pigmented cells in the control was constant throughout the culture period (Table 3.6.4). Both MS12%S and MS3%S+M were similar to the control for the first three days then increased between d.3 and d.24 and were

significantly higher than the control on d.24 (at P=0.001 and P=0.01 respectively). However, the proportion of pigmented cells for MS3%S+M was less than MS12%S on d.24 (significant at P=0.05). Therefore, increasing the osmolality of the medium increases the proportion of pigmented cells. These results also show that the increase in pigment for MS12%S and MS3%S+M is at least partly due to an increase in the number of pigmented cells since the proportion of pigmented cells either remained constant or increased as the total cell number increased. However, an increase in the amount of pigment per cell may also have occurred.

Changes in the sucrose concentration in the media (Table 3.6.5(a)) show that for the control the sucrose was constant between d.0 and d.3, decreased between d.3 and d.24 and reached *ca.* zero by d.24. The depletion of sucrose from the medium corresponds to the period when the cultures were growing. The sucrose concentration in the MS12%S treatment was constant throughout the culture period. Any changes in the MS12%S sucrose level were not significant. The MS3%S+M sucrose level was not significantly different from the control on d.0 and d.3. Between d.3 and d.24 the sucrose level for MS3%S+M decreased but the decrease was less than the control. Therefore, the sucrose concentration for MS3%S+M was higher than the control on d.24 (significant at P=0.01). These results show that when the osmolality of the medium was increased the sucrose was metabolized much less than in the control. This is to be expected since the cultures grew less when the osmolality was increased (see Table 3.6.1).

The results presented in Table 3.6.5(b) show changes in the osmolality of the medium during the culture period. The osmolality of MS12%S and MS3%S+M should have been similar on d.0 but MS12%S was significantly higher (at P=0.01). The osmolality of all the media increased when they were autoclaved but the osmolality of MS12%S increased more than MS3%S+M. Therefore, the value for MS12%S was higher than the value for MS3%S+M. For the control the osmolality increased 1.3 fold between d.0 and d.3 then decreased 8.1 fold between d.3 and d.24 as the cultures grew and sucrose was metabolized. The MS12%S treatment increased 1.5 fold between d.0 and d.24 whereas the MS3%S+M treatment increased 1.1 fold in the first three days then remained constant. Once again, the initial increase is probably due to the cells releasing metabolites as they equilibrate with the medium. This is similar to the previous experiment (3.5.3). MS12%S was higher than MS3%S+M throughout the culture period.

Overall, these results show that increasing the osmolality of the medium resulted

in a decrease in the growth of the cultures. However, it also resulted in an increase in the proportion of pigmented cells, pigment per g. fresh weight and cell number per g. fresh weight. Therefore, the cells were smaller and the number of pigmented cells was greater when the osmolality of the medium was raised. When the cultures started to grow the pigment per g. fresh weight decreased as in 3.5. Increasing the osmolality of the medium did not affect the viability of the cultures as it was *ca.* 78% for all three media on d.24 which is similar to the previous experiment (3.5.3). The cultures on MS3%S+M grew more than those on MS12%S. Since there appears to be an inverse relationship between growth and osmolality this increased growth on MS3%S+M was probably due to the initial osmolality of the MS3%S+M medium being lower than the MS12%S medium. It could also explain why there was less pigment per g. fresh weight for MS3%S+M. From these results the increased pigment levels at high sucrose concentrations appears to be due to the increased osmolality of the medium.

Table 3.6.1

Changes in the fresh weight of suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

Time (Days)	**Fresh Weight (g.)**		
	Control	**MS12%S**	**MS3%S+M**
0	0.449 ± 0.037	0.449 ± 0.037	0.449 ± 0.037
3	0.522 ± 0.078	0.466 ± 0.119	0.488 ± 0.077
24	7.338 ± 0.137	0.904 ± 0.089 ***	1.458 ± 0.225 ***

(significant from the control at *** P=0.001)

Table 3.6.2

Changes in total carotenoid pigment per flask (a) and per g. fresh weight (b) measured at 460nm in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

Table 3.6.2(a)

Time (Days)	**O.D. at 460nm. per flask**		
	Control	**MS12%S**	**MS3%S+M**
0	0.206 ± 0.006	0.206 ± 0.006	0.206 ± 0.006
3	0.327 ± 0.009	0.219 ± 0.019 **	0.249 ± 0.018 *
24	1.227 ± 0.088	1.597 ± 0.058 *	1.253 ± 0.047

(significant from the control at * P=0.05 and ** P=0.01)

Table 3.6.2(b)

Time (Days)	**O.D. at 460nm. per g. FW**		
	Control	**MS12%S**	**MS3%S+M**
0	0.462 ± 0.023	0.462 ± 0.023	0.462 ± 0.023
3	0.648 ± 0.075	0.551 ± 0.161	0.548 ± 0.120
24	0.168 ± 0.015	1.791 ± 0.134 ***	0.916 ± 0.190 *

(significant from the control at * P=0.05 and *** P=0.001)

Table 3.6.3

Changes in cell number per flask (a) and per g. fresh weight (b) in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

Table 3.6.3(a)

Time (Days)	Cell Number per flask x 10^{-6}		
	Control	**MS12%S**	**MS3%S+M**
0	3.910 ± 0.290	3.910 ± 0.290	3.910 ± 0.290
3	5.199 ± 0.931	5.174 ± 1.523	5.436 ± 0.706
24	42.670 ± 4.212	7.608 ± 0.994 **	11.456 ± 1.895 **

(significant from the control at ** P=0.01)

Table 3.6.3(b)

Time (Days)	Cell Number per g. FW x 10^{-6}		
	Control	**MS12%S**	**MS3%S+M**
0	8.731 ± 0.204	8.731 ± 0.204	8.731 ± 0.204
3	9.870 ± 0.279	10.923 ± 0.429	11.261 ± 0.392 *
24	5.798 ± 0.466	8.369 ± 0.337 *	7.821 ± 0.150 *

(significant from the control at * P=0.05)

Table 3.6.4

Changes in the proportion of pigmented cells in suspension cultures of *B.orellana* during batch growth.

Time (Days)	Proportion of Pigmented Cells (%)		
	Control	**MS12%S**	**MS3%S+M**
0	0	0	0
3	0	0	0
24	0	2.9 ***	1.3 **

(significant from the control at ** P=0.01 and *** P=0.001)

Table 3.6.5

Changes in the sucrose concentration (a) and osmolality (b) of the medium of *B.orellana* suspension cultures during batch growth. Each value is the mean of three replicates ± s.e.

Table 3.6.5(a)

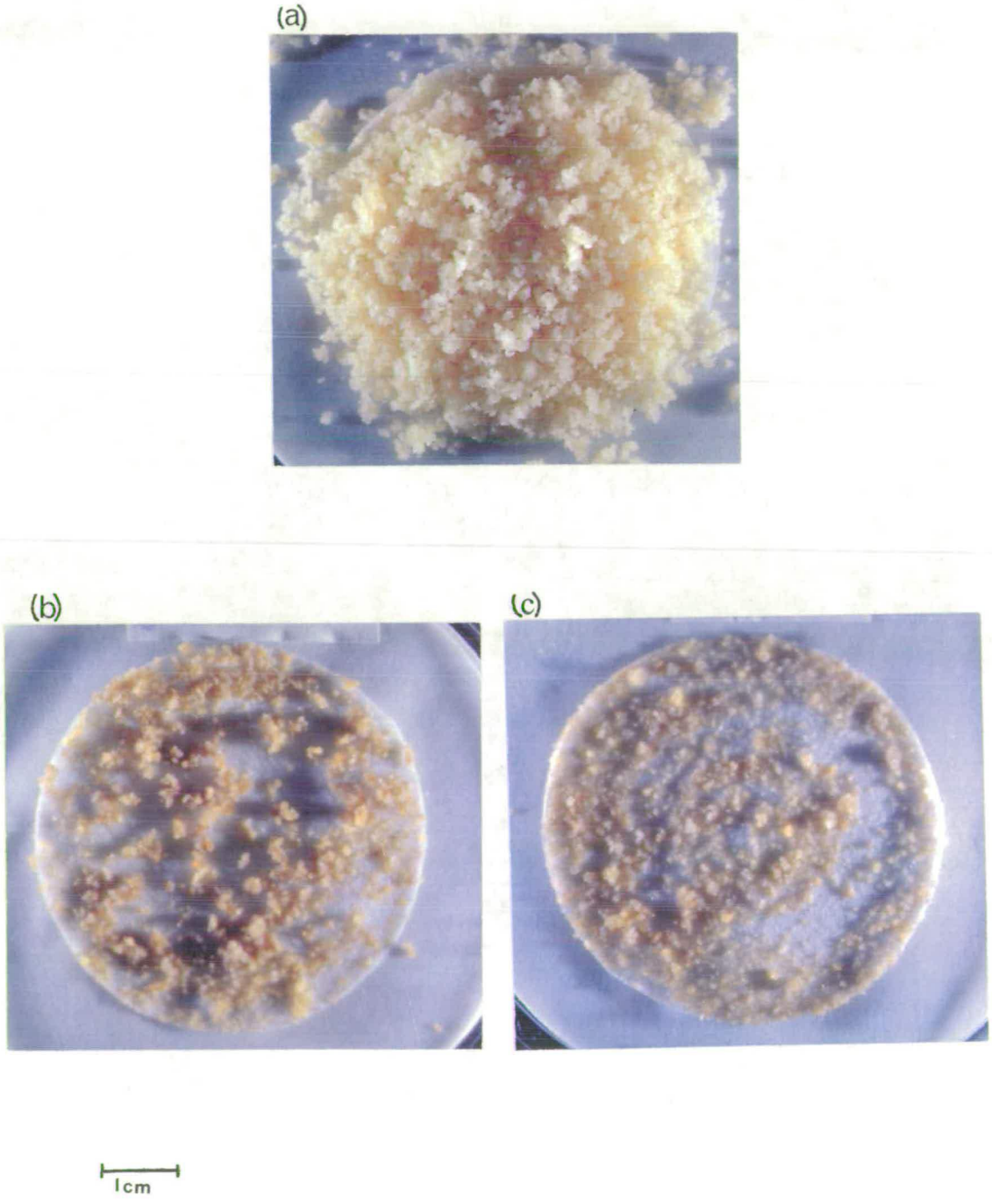
Time (Days)	Sucrose Concentration (mg.ml.$^{-1}$)		
	Control	**MS12%S**	**MS3%S+M**
0	29.67 ± 1.76	103.33 ± 8.33	27.67 ± 0.88
3	32.33 ± 2.03	123.33 ± 13.33	30.33 ± 0.67
24	0.16 ± 0.04	96.67 ± 16.91	15.67 ± 2.03

Table 3.6.5(b)

Time (Days)	Osmolality (osmol.kg.$^{-1}$)		
	Control	**MS12%S**	**MS3%S+M**
0	0.180 ± 0.001	0.477 ± 0.004	0.449 ± 0.004
3	0.227 ± 0.001	0.510 ± 0.005	0.471 ± 0.004
24	0.028 ± 0.004	0.697 ± 0.017	0.448 ± 0.011

Figure 3.6.1

The appearance of suspension cultures grown on (a) control, (b) MS12%S and (c) MS3%S+M media on d.24.



Summary of the Results in Section 3.6

The following points have arisen from this section.

(1) Increasing the osmolality of the culture medium resulted in a decrease in the growth of suspension cultures and an increase in pigment levels during the culture period.

(2) The cells became smaller and there was a rise in the number of pigmented cells when the osmolality of the culture medium was increased.

(3) Increasing the osmolality of the medium did not affect the viability of the cultures.

Therefore, the results in this section show that the higher pigment levels obtained when the sucrose concentration was raised above 3% appear to be due to the increased osmolality of the culture medium.

Chapter 4

Discussion

From the evidence available in the literature it would appear there are at least two different patterns of secondary metabolite accumulation in cultured plant cells (see Lindsey and Yeoman, 1985; Komamine *et al.*, 1989; Yeoman *et al.*, 1989). (1) Accumulation during the stationary phase which is usually associated with differentiation (eg. Lindsey and Yeoman, 1983; Bohm *et al.*, 1991). Here, there is an inverse relationship between culture growth and the accumulation of secondary metabolites (Yeoman *et al.*, 1980, 1982), and the production of most secondary metabolites falls into this category. (2) Accumulation of secondary metabolites during the active phase of growth (eg. Corduan and Reinhard, 1972; Kadkade, 1982; Hirose *et al.*, 1990), where there is a positive correlation between secondary metabolite production and culture growth. There are also reports of the accumulation of secondary metabolites during the lag phase of growth (Noguchi and Sankawa, 1982; Cvikrova *et al.*, 1988) but this could well be an extension of the accumulation associated with the downturn of growth after subculture.

Little is known about the regulation of precursor flow into primary and secondary metabolism. However, there is some evidence that cell growth rate may be linked to the regulation of secondary metabolism by affecting the kinetic partitioning of precursors between primary and secondary pathways (Yeoman, 1987). This may be achieved by changes in enzyme activities (Yeoman *et al.*, 1980) or alterations to enzyme/substrate compartmentalization (Lindsey and Yeoman, 1985). Indeed, availability of substrates is also likely to be involved since culture growth can be moderated by the level of substrate and this can result in increased secondary metabolism (Lindsey and Yeoman, 1983; Lindsey, 1985). In this discussion the production of carotenoids by cultures of *B.orellana* is considered in relation to growth and how this relationship can be exploited to improve product yield.

The discussion is divided into five sections. In the first the nature and distribution of carotenoid pigments in the plant is examined. The second section considers the establishment of callus and the attempts made to improve the level of pigmentation in these cultures. In the third section an alternative approach is discussed in which the fate of the pigment during callus initiation from root explants is examined to provide a means of enhancing pigment levels. Subsequently, the means to increase the production of carotenoid pigments during the batch growth of suspension cultures is assessed. Finally the discussion concentrates on the reasons why the use of nutrient stress by increased sucrose levels in the culture medium leads to an increase in pigment yield.

4.1 THE DISTRIBUTION AND NATURE OF CAROTENOID PIGMENTS IN THE PLANT

Much of the literature available on *B.orellana* is concerned with the annatto colouring obtained from seeds, especially its extraction (Preston and Rickard, 1980; Bhalkar and Dubash, 1983; Tong, 1984) and analysis (M^{c}Keown, 1961; Dendy, 1966; Tirimanna, 1981). Despite reports on the detailed anatomy of the plant (Metcalfe and Chalk, 1950) there are no reports on the distribution and nature of the pigments present in other parts of the plant.

4.1.1 The Distribution of Carotenoid Pigments in *B.orellana*

In this study it has been shown that orange/red pigmented cells are present in phloem and cortical tissues of stems, petioles and roots of *B.orellana* and in the phloem and spongy mesophyll of leaves (see 3.1.1). The fact that the pigmented cells are widely distributed throughout the plant suggests that the pigment is produced locally and not synthesized in one area then transported to another. The proportion of pigmented cells is similar in the leaves, stems, petioles and roots but within the tissues the cells are randomly distributed (see 3.1.2). This widespread, random distribution suggests that the pigment has a general function which is not known but the fact that it is present in the roots and not just the photosynthetic parts of the plant indicates that its function is not just in photosynthesis or as a photoprotectant. In all of the tissues the pigmented cells are parenchymatous. This is not surprising since such cells function in storage, photosynthesis and sometimes secretion.

The pigment within the cells has a very granular appearance (see Fig. 3.1.4) and when the cells are damaged it forms regular shaped droplets which do not mix with water. This suggests that the pigment is oil based. The granular appearance of the pigment is consistent with its restriction to chromoplasts, carotenoid containing plastids. Such plastids are found in large numbers in flowers, fruits and roots and there are reports that these organelles are derived from chloroplasts, proplastids and amyloplasts (Kirk and Tilney-Bassett, 1978). In plants the biosynthesis of carotenoids is restricted to plastids eg. chromoplasts of fruit and flowers and chloroplasts of photosynthetic tissue (Britton, 1988). This restriction and the widespread distribution of pigmented cells suggest that the pigment is produced locally within the plastids of certain cells. Which cells become pigmented seems to be a random process within the tissue but there appears to be some control over the proportion of pigmented cells since this is constant in the different organs of the plant.

4.1.2 The Nature of Carotenoid Pigments in *B.orellana*

When plant pigments other than those of the seeds were analysed, bixin along with several other carotenoids including β-carotene and the xanthophyll lutein were shown to be present (3.1.3). Carotenoids such as β-carotene and lutein are found in almost all plants (see Goodwin, 1980; Goodwin and Britton, 1988) but bixin is unique to *B.orellana*. There are no reports of bixin in any other plant. Seven of the pigments in the plant were not identified. One of these is an unknown yellow compound(s) with absorption maxima at 419-420, 398-401 and 375-380nm. (in chloroform) which co-chromatographs with bixin. Attempts to separate and identify this compound were not successful but it could be similar to one of the yellow compounds found in annatto seed extracts, for example, orellin or a breakdown product of bixin (McKeown, 1961, 1965; Tong, 1984). Both of these have a similar λ_{max} to this unknown yellow compound found in the leaves, petioles, stems and roots (McKeown, 1961). The pathway for bixin formation is not known but this unknown yellow compound could be a precursor for bixin or a breakdown product of bixin.

When the pigment layer surrounding the seeds of *B.orellana* was extracted only bixin was shown to be present (see 3.1.3). However, this is not in agreement with reports in the literature, for example, McKeown (1961) found that the seeds contained seven pigments. The number and concentration of pigments present in seed extracts especially those of the minor constituents may depend on the extraction procedure, the temperature of extraction, the time required for extraction and the origin and age of the seeds (Preston and Rickard, 1980). In this study the seeds that were extracted were *ca.* 2 years old and the method of pigment extraction was different to that of McKeown (1961) so these factors may have affected the results.

It seems clear that bixin is present in leaves, petioles, stems, roots and seeds of *B.orellana* with the highest levels in the seeds. The reason for this is unknown. Many of the carotenoids found in plants function in photosynthesis. Although the function of bixin is not known it is unlikely that it acts in photosynthesis or as a photoprotectant since it is present in non-green organs and no other plant contains such a pigment. The wide distribution of pigmented cells and chromoplasts in *B.orellana* suggests that the pigment has a more general function. When the plant is wounded the orange pigment moves to the wound site but in the sections examined there was no evidence of channels or specialized cells for the pigment to move in. In addition it was noted that movement of the pigment was especially obvious in tissues from young seedlings. One possibility is that bixin may have a dual role: (a) it could

protect the plant from grazing predators especially at an early stage of development, thereby allowing the plant to reach maturity and (b) once mature, animals could be attracted to the seeds. This would aid in their dispersal and ensure propagation of the plant. Another possibility is that bixin is stored in the plant for later transport to the seeds.

Whatever its function in the plant, bixin is a very important colourant in the food industry. Having found that the stems, leaves, petioles and roots of *B.orellana* all contain bixin, the production of carotenoid pigments in cultures of this plant were examined. Establishment of cultures of *B.orellana* and carotenoid pigment production in these cultures are now discussed.

4.2 THE OCCURRENCE AND IMPROVEMENT OF PIGMENT YIELD IN CALLUS CULTURES

In order to study carotenoid production *in vitro* it is necessary to establish cultures of *B.orellana*, choose cultures which produce pigment and use a culture method which is likely to promote secondary metabolite production. The establishment of callus cultures and attempts to improve the pigment levels in these cultures are considered in this section.

4.2.1 Establishment of Callus Cultures of *B.orellana*

The majority of explants require an exogenous supply of auxin and/or cytokinin for growth and cell division when placed in culture (Dougall, 1980). The amounts of these plant growth regulators added to the culture media are critical in determining the extent of growth and the incidence of organogenesis (Skoog and Miller, 1957). In this study organogenesis was minimized and callus growth maximized by optimizing and balancing the amounts of plant growth regulators in the culture medium. In general, the addition of a cytokinin to the culture medium prevented root regeneration and encouraged the formation of white, friable callus (see 3.2.1.1). Optimum growth rates were obtained when the cytokinin added to the culture medium was 2-IP (3.2.1.2). In preliminary studies the only cytokinin present in the medium was provided by coconut milk (see Table 3.2.1) but here the concentration was too low to sustain good callus growth since it promoted root formation. Low levels of cytokinin along with high amounts of auxin in the culture medium promote root growth in tobacco callus cultures (see Skoog and Miller, 1957). Therefore, the addition of cytokinin to the culture medium would alter the balance of growth regulators in favour of callus growth.

The balance between auxin and cytokinin concentration is crucial in establishing and maintaining cultures. Optimum mean relative growth rates were obtained with two media: MS medium with 5 x 10^{-7}M 2-IP, 2.7 x 10^{-6}M NAA and MS medium with 1 x 10^{-6}M 2-IP, 5.4 x 10^{-6}M NAA (see 3.2.1.3). Reducing the length of the culture period from 28d. to 14d. also increased the mean relative growth rate of the cultures (see Table 4.1). The length of the culture period in tissue culture is variable and depends on the rate of callus growth (Dodds and Roberts, 1985). In this study the nutrients in the medium would probably have been exhausted during the longer culture period, therefore resulting in cessation of cell division and growth and leading to an overall reduction in growth rate.

Table 4.1

Mean relative growth rates for callus cultures grown on MS medium with 5 x 10^{-7}M 2-IP and 2.7 x 10^{-6}M NAA for three different culture periods during the first subculture after initiation. Each value is the mean of three replicates ± s.e.

Time of Culture Period (d.)	**Mean Relative Growth Rate ($d.^{-1}$ x 10^2)**
14	6.27 ± 0.39
21	4.18 ± 0.95
28	1.37 ± 0.19

It was also established that MS medium was the best nutrient medium for callus growth (3.2.1.4). This is perhaps not surprising since MS medium is widely used in tissue culture (see Gamborg and Shyluk, 1981). Callus tissue was produced readily from roots, leaves and stems of *B.orellana* (3.2.1.5). The callus from leaves was hard with no pigmented cells whereas white, friable callus containing some orange pigmented cells was obtained from both root and stem explants. This result is

consistent with evidence in the literature which suggests that different parts of an individual plant can at least initially give rise to cultures which display differences in secondary metabolism, for example in the levels of lignanes (Kadkade, 1982). However, the successful establishment of callus depends largely on the culture conditions employed and not on the source of plant material (Yeoman and Macleod, 1977). Therefore, the reason that hard callus is produced from leaf tissue is probably because of the culture conditions. As culture initiation depends partly on the developmental state of the tissue, for example, variation in the proliferative capacity of explants of pith tissue from different regions of the tobacco plant was due to the cytokinin requirement of the pith tissue changing along the length of the stem (Meins, 1986), it is expected that tissues from different developmental states will not respond uniformly. The response of an explant in culture, therefore depends to some extent on the endogenous growth substances present at the time of excision (Yeoman and Macleod, 1977). The reaction obtained from the explant may also depend to varying extents on the condition of the parent plant as affected by the season, growing conditions and age (Hussey, 1986). Therefore, different tissues from the same plant would be expected to have different requirements for callus initiation depending on their developmental state at the time of excision. This may also affect the production of secondary metabolites, as secondary metabolism is claimed to be related to growth and organogenesis (see Yeoman *et al.*, 1980, 1982; Yeoman, 1987).

The pigment levels in callus cultures of *B.orellana* were very low and appeared to decline as the cultures aged. This low production of secondary metabolites is a general problem in plant tissue culture and, with few exceptions, cultures accumulate lower levels of secondary metabolites than the plant (see Fowler, 1986; Collin, 1987; Wink, 1990). In this instance the level of pigment in the cultures was too low to be extracted and analysed so attempts were made to increase the levels.

4.2.2 Attempts to Improve Pigmentation in Callus Cultures

It has been shown that yields of secondary metabolites can be increased by making modifications to the culture medium, to the culture conditions and also by selecting for high-yielding clones (see Collin, 1987). Generally, the media used for the culture of callus and suspension cultures of higher plants were originally devised to support rapid growth. Since secondary metabolite yield is often inversely related to growth, it is likely that these media will not be optimal for secondary metabolite production. In general, medium conditions which promote secondary metabolism are those which limit cell division and lead to a comparatively early cessation of

exponential growth (Mantell and Smith, 1983). This suggests that the culture conditions have to be altered to promote secondary metabolism.

There are many reports where altering the culture medium increases secondary metabolite production. For example, depletion or deficiency of nitrogen and phosphate is associated with growth limitation and concomitant secondary metabolism (see Knobloch and Berlin, 1980, 1981; Yamakawa *et al.*, 1983; Lindsey, 1985). Similarly low auxin concentration, particularly of 2,4-D, favours secondary metabolism (see Collin, 1987). Instances have been reported where maximum yields of secondary metabolites were obtained when cells were raised on media devoid of auxin, and in many cases omission of auxin resulted in culture differentiation and morphogenesis (Mantell and Smith, 1983). Further it has been reported that the presence of picloram caused a substantial increase in the carotenoid content and a change in the carotenoid composition in *Allium cepa* callus cultures (Musker *et al.*, 1988). Here, picloram appeared to have two different effects, one on tissue differentiation and the other on cell structure and development. In general, these treatments usually limit growth and primary metabolism. This in turn tends to favour differentiation and secondary metabolism suggesting that there is an inverse relationship between growth and secondary metablite production (see Yeoman *et al.*, 1980, 1982).

When the culture medium was modified, by the addition of picloram (see 3.3.1), a reduction in the NAA concentration (see 3.3.2) or the removal of i-N and/or i-P (see 3.3.3), the growth rate and/or the viability of the *B.orellana* callus cultures decreased. However, none of these treatments promoted the synthesis and accumulation of pigment(s). This could have been due to two main reasons:

(1) The viability of the cultures decreased and this would affect their ability to produce pigment.

(2) Pigment production may not be inversely related to growth.

It has been shown that cells which accumulate anthocyanins at the end of one growth cycle are capable of further rounds of cell division (see Hall and Yeoman, 1986b). This shows, at least in that case, cells which accumulate secondary metabolites are viable. Also as explained earlier the accumulation of secondary metabolites is not necessarily related inversely to growth since there are a number of reports of secondary metabolite production during active growth of cells (see Collin, 1987; Lindsey and Yeoman, 1985). Therefore, it would seem that one or both of

these reasons could account for the lack of pigment in *B.orellana* cultures.

In a further attempt to increase the carotenoid level in callus cultures groups of pigmented cells were selected using cell-aggregate cloning together with the filter paper raft-nurse culture technique. The importance of the selection of high-yielding lines in the development of a system for secondary metabolite production *in vitro* has been stressed repeatedly (see Zenk, 1978; Yamada and Fujita, 1983; Collin and Dix, 1990). This approach, which has been used to obtain cell lines producing pigments including chlorophyll, carotenoids, anthocyanin and other pigment classes (Dix, 1986), was not successful in this study. The cell aggregates either died because the pieces of tissue selected were too small, or a callus was produced which consisted of predominantly non-pigmented cells. The predominance of non-pigmented cells could be due to:

(1) Pigmented cells not dividing

(2) Pigmented cells dividing but at a much slower rate than non-pigmented cells

(3) Daughter cells of pigmented cells not producing pigment

(4) Conditions of culture not promoting pigment accumulation

One possible explanation for the presence of pigment in newly initiated callus is that it is carried over from the explant which has pigmented cells. Then if the callus does not have the ability to produce pigment and turnover of pigment occurs, the total pigment in the callus will decline as the culture ages.

In this study the use of standard culture procedures did not improve the levels of carotenoid pigments in callus cultures of *B.orellana*. Clearly there are several possible reasons why the pigment levels in the cultures were not increased but which, if any, of these apply is not known.

4.3 THE FATE OF CAROTENOID PIGMENTS DURING CALLUS INITIATION

Attempts to increase pigment production in callus cultures of *B.orellana* using standard procedures such as reduction of auxin, removal of i-N and/or i-P, addition of picloram and selection methods were not successful (3.3). Therefore, an alternative approach was adopted in which changes in the carotenoid pigments during callus initiation from root explants were examined. Root tissue has pigmented cells and this

approach allowed the fate of this pigment during callus initiation to be determined.

4.3.1 Changes in Carotenoid Pigment Levels During Callus Initiation

The evidence presented in 3.4.1 and 3.4.2 showed that carotenoid pigment levels increased in root and callus tissue during callus initiation from root explants and the pattern of pigment accumulation during the culture period was similar for both callus and root. This would indicate a continuing accumulation of pigment in the root but the pigment which appears in the callus could either be produced *de novo* or transported from the root to the callus. It has already been pointed out that pigment moves when the explants are cut (see 3.1) so it is possible that transport of pigment may occur from root to callus. The data show that the proportion of pigmented cells in the root and callus either remain constant or increase during the culture period which suggests that the rise in the amount of pigment is at least partly due to an increase in the number of pigmented cells since the total cell number is increasing. Also the root tissue contained more pigment on a per mg. fresh weight basis (*ca.* 10 fold) than the callus. This is partly due to a greater number of pigmented cells since the proportion of pigmented cells is higher in the root. The evidence reported here is consistent with that reported by Hall and Yeoman (1987) who found that variation in accumulation of anthocyanin in *Catharanthus roseus* cultures was primarily due to differing proportions of pigmented cells rather than mean intracellular anthocyanin concentrations within the cells. The pigment changes in *B.orellana* callus can largely be accounted for by differences in the number of pigmented cells. However, increases in the amount of pigment per cell may also occur, but this was not determined in this study.

Pigment increases were observed in both the root and callus despite the fact that the tissues were growing at different rates. Therefore, it is difficult to predict from these results if there is a relationship between growth and pigment production. However, the fact that the root contains more pigment on a fresh weight basis than the callus may be related to the slower growth of the root tissue. If this is so, it suggests an inverse relationship exists between growth rate and overall pigment content of the tissue. The existence of such a relationship is not surprising since the production of most secondary metabolites is inversely related to growth (Yeoman *et al.*, 1980, 1982).

As callus is initiated there is a decrease in the average cell size in the root and the cells in the callus were generally larger than those in the root. The decreasing cell

size is due to cell division taking place more rapidly than cell growth. Indeed it is the balance between division and growth which affects the final average cell size within the callus (Aitchison *et al.*, 1977). However, the cells in the callus appear to be dividing faster than the root, therefore since the callus cells are larger than those of the root there must have been considerable cell expansion in the cells of the callus. These differences in the cells may have affected pigment accumulation in the tissues. Indeed, it has been suggested that the physiological state of the cell partly determines the capacity to synthesize secondary metabolites (see Collin, 1987).

From this study, it appears that pigmented cells are present in newly initiated callus tissue and the number of these cells increases during callus initiation. However, it is not known whether this pigment is carried over from the root tissue or if pigment is produced *de novo* and is related to growth. If a means can be found to separate growth and pigment production the relationship between the two can be studied further.

4.3.2 The Effect of Nutrient Stress on Carotenoid Pigment Levels During Callus Initiation from Root Tissue

It has been shown by a number of workers that most rapidly growing cultures do not accumulate secondary products to any great extent because the precursors of the desired product are required for the primary metabolic processes essential for growth and division (Hall and Yeoman, 1986a). Indeed evidence in the literature suggests that there is an inverse relationship between culture growth and the production of many secondary metabolites (see Yeoman *et al.*, 1980, 1982). There are also many reports showing that the application of nutrient stress results in a decrease in growth of cultures and an increase in secondary metabolism (Mantell and Smith, 1983; Collin, 1987).

Manipulating the culture medium by the use of 8% instead of 3% sucrose or removing i-N and/or i-P decreased the growth of newly initiated callus tissue and increased the pigment on a per mg. fresh weight basis compared to the control (see 3.4.3). This suggests that there is an inverse relationship between growth and carotenoid production. These treatments have all been reported to increase secondary metabolism in cultures (see Collin, 1987; Mantell and Smith, 1983). The reduced growth with 8% sucrose may have been due to an alteration in the C/N ratio comparable to the effect of removing i-N while keeping the level of the carbon supply constant. This ratio may also, indirectly through growth, affect the production of secondary metabolites. Indeed Yamakawa *et al.* (1983) reported that the C/N ratio is

important in determining the extent of secondary metabolism. In this study the higher pigment levels for the treatments compared to the control were not observed until the end of the culture period (d.28), so the effect was only apparent after a long period in culture. These higher pigment levels with the treatments may have been due to increased pigment production as a result of reduced growth. If this is so, an inverse relationship must exist between carotenoid pigment production and growth. The MS-N and MS-NP treatments had stopped growing by d.28 but the MS8%S and MS-P treatments were still growing. There was no cell division in the callus at this time so any increase in fresh weight must have been due to the expansion of existing cells. However, there was no apparent increase in the average cell size in the callus at the end of the culture period but this could have been masked by the large sampling errors. The production of anthocyanin during cell expansion prior to the stationary phase has been shown to occur in *Catharanthus roseus* cultures (Hall and Yeoman, 1986a). Such production may also be occurring in *B.orellana* cultures but it is not clear from this study.

In the callus tissue there was an increase in carotenoid pigment on a fresh weight basis during the first 12d. of culture for all of the treatments, including the control, so pigmented cells were produced as callus was initiated. This increase along with the higher pigment levels towards the end of the culture period suggests that possibly the pigment is produced at a greater rate just before and after cell division but evidence for such production is not conclusive from this study. Such a pattern of accumulation occurs in *Salvia miltiorrhiza* where ferruginol, a diterpene, accumulated during the lag phase, the final part of the exponential phase and during the stationary phase (Miyasaka *et al.*, 1985). The biphasic pattern was explained by the fact that secondary metabolism occurred at a time when the primary pathways were relatively inactive.

With root tissue higher pigment levels on a fresh weight basis compared to the control also occurred for the MS8%S, MS-N and MS-NP treatments towards the end of the culture period. However, the increased pigment levels were less than those for callus. There was no initial rise in the root pigment but this may have been masked by the large initial decrease probably due to loss of pigment at the cut surface of the explant as it moved to the cut surface when explants were cut. A gradient of nutrients was probably quickly established in the culture medium as nutrients are depleted by the growing callus. The extent of this gradient will depend on the amount of callus growth so that less nutrients will be available for the root tissue as the callus increases in size. Since the production of secondary metabolites depends on the precursors

available to the tissue, a limitation to the supply of these precursors in the root could explain low pigment production. Low levels of precursors in the root could also explain the larger pigment increases in the callus than in the root. These differences between root and callus are consistent with no carryover of pigment to callus from the root.

As already stated, the cells of the callus were generally larger than those of the root explant. On MS8%S the cell number and cell size were similar to the control but on MS-N, MS-P and MS-NP the cell number and cell size were lower. This suggests that MS8%S has a different effect on the cells and possibly pigment production when compared to the other three treatments. The proportion of pigmented cells either remained constant or increased during the culture period for all treatments. Since there was an increase in cell number there must also have been a rise in the number of pigmented cells as the proportion of pigmented cells remained constant. Therefore, any increases in pigment were at least partly due to increases in the number of pigmented cells.

These results suggest that there is an inverse relationship between growth and pigment production. However, the usefulness of callus as an experimental system is limited because it has a relatively slow rate of growth, consists of cells which vary considerably in age and is subject to nutrient gradients which develop as nutrients are depleted (Gamborg and Shyluk, 1981). The correlation of secondary metabolism with a specific culture growth phase can be made more easily using a suspension culture which has the advantage that nutrients can be continually adjusted and the cells are surrounded by the medium. Here altering the culture medium has a more direct effect on the cells than with callus.

4.4 CAROTENOID PRODUCTION DURING BATCH GROWTH OF SUSPENSION CULTURES

In order to study carotenoid production in a suspension culture system it was first necessary to establish the cultures and characterize their growth and pigment production. The establishment of a suspension culture system and improvement of pigment production in such a system are considered in this section.

4.4.1 Establishment and Characterization of Batch Growth of Suspension Cultures

Much of the evidence in the literature on physiological and biochemical investigations of growth and cell division in plant cell cultures has been obtained

from batch cultures (King and Street, 1977). In such batch propagation the culture shows a growth cycle which takes the generalized form of a sigmoidal curve and consists of several phases (see Lindsey and Yeoman, 1985). The occurrence and duration of each phase depends very much on the species, the frequency of subculture, the inoculum and the culture medium (King and Street, 1977).

Suspension cultures of *B.orellana* were established on MS medium containing 1 x 10^{-6}M 2-IP and 5.4 x 10^{-6}M NAA and the optimum inoculum density for initiation and growth of these cultures was 1.0g. of friable callus tissue and 1.0g. of filtered suspension culture cells respectively (see 3.2.2.1 and 3.2.2.2). The inoculum size is extremely important in batch culture as there is generally a minimum size below which the culture will not grow (Gamborg and Shyluk, 1981). The size of the inoculum also determines the lag period. Using a 1.0g. inoculum the batch growth of suspension cultures was shown to consist of a lag phase of *ca.* 4d. when the cells were adapting to the fresh medium with no cell division or growth (see 3.2.2.3). Cell division was evident after 4d. and continued until d.16. Division ceased after 16d. but cell expansion continued until d.20. The stationary phase was reached by d.20 and there was no further cell expansion or increase in cell number probably due to lack of essential nutrients in the culture medium.

During such batch growth it has been shown that secondary metabolites often begin to accumulate at a distinct period of the growth cycle and the accumulation is dependent on factors such as age and size of the inoculum (King and Street, 1977).

4.4.2 Changes in Pigment Levels During Batch Growth

Reports in the literature show that in most cases, but not all, secondary metabolites tend to be accumulated in suspension cultures late in batch growth after the cessation of cell division and they are associated with cell differentiation (Lindsey and Yeoman, 1985; Collin, 1987; Yeoman *et al.*, 1989). There are, however, exceptions where the product is produced during growth eg. phenolics in cell suspensions of Paul's Scarlet rose (Nash and Davies, 1972), germichrysone in suspension cultures of *Cassia torosa* (Noguchi and Sankawa, 1982) betacyanin in suspension cultures of *Phytolacca americana* (Sakuta *et al.*, 1986) and in *Beta vulgaris* (Mohd. Shaib, personal communication).

In *B.orellana* suspension cultures carotenoid pigment(s) was present and this increased during the culture period (see 3.5.1). Increases in pigment per flask occurred in the lag and early growth phases of batch growth. However, there was

only an increase in pigment per g. fresh weight during the lag phase. Thus, as the cell number increased pigment was produced but the pigment per g. fresh weight decreased. This could have been due to:

(1) Pigmented cells are produced but at a slower rate than non-pigmented cells and the pigmented cells are 'diluted out'.

(2) An increase in the pigment per cell with no increase in the number of pigmented cells.

(3) A combination of (1) and (2).

Unfortunately no pigmented cells were detected in suspension cultures of *B.orellana.* However, measurable amounts of pigment were extracted from the cultures so pigmented cells must be present. Indeed, pigmented cells were occasionally observed in the cultures using an inverted microscope. This apparent inconsistency is probably due to the numbers of pigmented cells in the cultures being very small. Therefore, whether the changes in pigment were due to changes in numbers of pigmented cells could not be determined. Several factors could have contributed to the increase in pigment:

(a) An increase in pigment per cell

(b) Non-pigmented cells become pigmented

(c) New cells produced by division are pigmented

During the later stages of growth the pigment remained constant. The fact that there was no decrease in pigment per flask suggests that there was no overall breakdown of pigment, however, turnover of pigment may have been occurring.

In the literature there are several reports of carotenoid production in carrot cell cultures occurring in parallel with growth (Sugano *et al.*, 1971; Mok *et al.*, 1976; Shimizu *et al.*, 1979). Shimizu *et al.* (1979) reported that carotenoids were most actively synthesized in the early logarithmic phase of growth and the synthetic rate declined sharply as the culture aged. However, there was no apparent lag phase in the growth of these carrot cultures so it is not known if pigment production occurred during this phase. Production of carotenoids during the early growth of cultures is consistent with the findings with *B.orellana.*

It is reported that the lag phase of growth is characterized by intensive metabolic activity and that the balance of metabolism is directed towards biosynthesis (see Fowler, 1971; Nash and Davies, 1972; Shimizu *et al.*, 1977). For example, enzyme activities relating to carbohydrate metabolism increase during the lag phase (Fowler, 1971) and there are major changes in the level of NADPH, ATP and energy charge (Shimizu *et al.*, 1977). Once cell division has commenced there is a gradual change in the pattern of metabolism (Shimizu *et al.*, 1977). Such metabolic changes may be involved in the production of carotenoid pigment in *B.orellana* suspension cultures since it is produced during the early stages of growth.

The induction of carotenoid production in *B.orellana* could be due to:

(1) A 'dilution effect' caused by the subculture of cells into fresh medium.

(2) Changes in carbon metabolism related to plastid development.

Increases in enzyme activity, especially phenylalanine ammonia-lyase (PAL), have been reported in the lag phase of culture and are considered to be due to a 'transfer effect' or 'dilution effect' when the cells are subcultured (see Hahlbrock and Wellmann, 1973; Hahlbrock and Schroder, 1975; Bevan and Northcote, 1979; Dixon *et al.*, 1980; Ozeki *et al.*, 1987; E. Groskurt, personal communication). This dilution effect may be the result of lowered intracellular concentrations of pathway intermediates (see Dixon *et al.*, 1980). In carrot cell suspension cultures PAL synthesis was induced after transfer to fresh medium and during anthocyanin production which took place after cell division had ceased and it was suggested that two different PAL genes were involved (Ozeki *et al.*, 1990). Therefore, similar events may be involved in carotenoid pigment production in cultures of *B.orellana.* The production of germichrysone is thought to be induced by two such mechanisms in cultures of *Cassia torosa* (Noguchi and Sankawa, 1982).

As mentioned previously carotenoid production in *B.orellana* suspension cultures occurs during the early stages of batch growth. For this to happen there must be a stimulus to allow differentiation of chromoplasts and therefore, pigment production to occur. In the plant, it is generally accepted that plastids arise from pre-existing plastids (Possingham, 1980). They can arise either by interconversion of one type of plastid into another or by division (see Possingham, 1980; Thomson and Whatley, 1980). Both chloroplasts and proplastids are known to multiply by division and are transmitted from cell to cell (Kirk and Tilney-Bassett, 1978; Possingham, 1980; Mullet, 1988). Clearly something must trigger the development of particular plastids.

The various plastids contain 'plastid specific' proteins, the formation of which requires a number of enzymes. Thus the formation of this range of plastids must involve the activation of different genes. It is known that plastid differentiation is under the control of both plastid and nuclear genes (Kirk and Tilney-Basset, 1978; Taylor, 1989) and that chloroplast development is predominantly influenced by light. Indeed, blue light has been found to have a strong stimulatory effect on the transcription of nuclear and plastid genes (Kaldenhoff and Richter, 1990). However, the development of chloroplast structure is also affected by other factors, particularly hormones, nutritional state and water stress (Sundqvist *et al.*, 1980; Mullet, 1988). Unlike the differentiation of chloroplasts the nature of chromoplast differentiation is not understood, but must occur early in the batch growth of *B.orellana* suspension cultures to enable carotenoid production to occur.

It is still not known whether carotenoid production is related directly to the growth of the cultures or how it is controlled. However, chloroplast development depends on the nutritional state of the plant, therefore the nutritional state of the culture is likely to be important in determining chromoplast development in suspended cells.

4.4.3 The Effect of Nutrient Stress on Pigment Production in Suspension Cultures

There are numerous reports in the literature of how the application of nutrient stress to plant tissue cultures can decrease growth and primary metabolism and promote differentiation and secondary metabolism (see Mantell and Smith, 1983; Collin, 1987). As already mentioned such nutrient stress may also affect plastid development. Previously nutrient stress was found to increase pigment production during callus initiation from root tissue of *B.orellana* and the production appeared to be inversely related to growth (see 4.3.2). The greatest effects were obtained when the culture medium contained 8% instead of 3% sucrose or was lacking i-N. When such treatments were tested on *B.orellana* suspension cultures pigment was produced during the lag and early phases of growth for both the control and the MS8%S, MS-N and MS8%S-N treatments (see 3.5.2).

As expected, the overall fresh weight and cell number of suspension cultures was much less in a culture medium lacking i-N. However, the pigment per g. fresh weight was higher than the control during the lag and stationary phase. The fact that there was less growth on MS-N would probably have lead to less dilution of the pigmented cells with non-pigmented cells and thus resulted in a higher level of pigment on a

fresh weight basis in the stationary phase. This could also account for the higher level of pigment at the end of the culture period during callus initiation from root tissue on a stress medium (see 4.3.2). The reason(s) for the increased pigment production during the lag phase on a per flask and a fresh weight basis when the medium was lacking i-N is not understood but it may be that removal of i-N encourages plastid development or changes metabolism but in a way not related to growth since the length of the lag phase was not altered.

As already mentioned there are numerous reports in the literature where reducing the nitrogen concentration in the culture medium increases secondary metabolite production (see Yamakawa *et al.*, 1983; Lindsey, 1985; Do and Cormier, 1991a). In the majority of cases a reduction in nitrogen was found to reduce the growth of the cultures and bring about a premature stationary phase. There is also a report of stimulated betacyanin accumulation accompanying an increase in total nitrogen concentration in suspension cultures of *Phytolacca americana* (Sakuta *et al.*, 1987) but in this case there is a close correlation between growth and betacyanin accumulation under normal growth conditions (Sakuta *et al.*, 1986). With *B.orellana* cultures i-N depletion slowed down cell division and growth but did not affect carotenoid production during the period of cell division or the stationary phase. This suggests that carotenoid production is not related to cell division. The fact that omitting i-N did not increase pigment production in the stationary phase of growth in suspension cultures is not surprising since it was previously shown that pigment production occurs in the early stages of batch growth. Therefore, bringing about a premature stationary phase by a reduction in i-N does not increase pigment production.

When the culture medium contained 8% instead of 3% sucrose the growth of suspension cultures was delayed and the lag phase was prolonged. The carotenoid pigment, per flask and per g. fresh weight, was then increased. This is not surprising since the early stages of culture are extended and this is when pigment production occurs. There are many reports in the literature of increased sucrose levels promoting secondary metabolism. For example, in the production of the steroidal alkaloid, solasodine, in callus cultures of *Solanum nigrum* (Bhatt *et al.*, 1983) and in suspension cultures of *Solanum eleagnifolium* (Nigra *et al.*, 1990), alkaloid and polyphenol production in dark grown suspension cultures of *Catharanthus roseus* (Knobloch and Berlin, 1980) and anthocyanin production in *Vitis* suspension cultures (Yamakawa *et al.*, 1983; Cormier *et al.*, 1990; Do and Cormier, 1991a). The increased accumulation of anthocyanin in *Vitis vinifera* suspension cultures when the

sucrose levels were raised has been shown to be the result of osmotic stress (Do and Cormier, 1990, 1991b). However, apart from these findings, most of the reports give little indication as to how the metabolism of the cells is affected by increased sucrose levels. Sucrose may have a similar effect in all cases or have a different effect depending on the species and the secondary metabolite.

In this study it was found that the increase in pigment on the MS8%S medium was partly due to an increase in the number of pigmented cells since the proportion of pigmented cells increased. However, there may also have been an increase in the pigment per cell. This is similar to previous findings when callus was initiated from root tissue. Also in this study there was an indication of pigment breakdown as the cells aged since the pigment per flask decreased for both the cultures on MS8%S and the control in later stages of batch growth (late linear and stationary phases). This is consistent with reports by Shimizu *et al.* (1979) who found that there was turnover of newly synthesized carotenes during the growth of carrot cell cultures.

When the medium contained 8% sucrose but did not contain i-N the growth of the suspension cultures was attenuated. Here, the absence of i-N prevented the culture attaining a biomass level similar to the control or cells on MS8%S media. Also omitting i-N prevented the prolonged increase in carotenoid pigment production observed with MS8%S. This may be because lack of i-N brings about a premature stationary phase of growth, a phase when pigment production does not occur in *B.orellana*. Increasing the sucrose and removing i-N from the culture medium alters the C/N ratio. Yamakawa *et al.* (1983) reported that increased anthocyanin levels occurred in *Vitis* cell cultures when sucrose levels were raised. However, they also found that at lower sucrose concentrations reduced nitrogen levels also resulted in higher amounts of anthocyanin and suggested that there was an optimal C/N ratio for pigment production.

One conclusion from this study is that lack of i-N attenuates cell division and thus brings about the stationary phase of growth. However, bringing about the stationary phase did not affect pigment production. From this it would appear that carotenoid production is not directly related to cell division. On the other hand, an increased sucrose level delayed growth, prolonged the lag phase and promoted pigment production and the largest increase in carotenoid production occurred when the sucrose concentration in the culture medium was raised. These findings support the view that carotenoid production in *B.orellana* suspension cultures occurs in the lag phase prior to cell division. The fact that pigment production occurs during the early

increase in cell number is probably due to the heterogeneous nature of a cell population in which not all of the cells are dividing at the same time. As the cell number increases and a greater proportion of the cells divide the lag phase will terminate and overall production will cease. Increasing the pigment level by prolonging the lag phase is also suggestive of an inverse relationship between growth and pigment production. However, this relationship is unlike many examples described in the literature since it occurs in the lag and not in the stationary phase of growth. This suggests that slow growth is related to secondary metabolism but is not the only trigger for such metabolism. Indeed, other factors must be involved such as the availability of precursors and plastid development. Clearly, a number of interacting factors are involved in the regulation of secondary metabolism.

4.5 THE NATURE OF THE 'SUCROSE EFFECT' IN SUSPENSION CULTURES OF *B.ORELLANA*

There are numerous reports in the literature of the promotion of secondary metabolism by increased sucrose levels in the culture medium (eg. Zenk *et al.*, 1977; Knobloch *et al.*, 1982; Roper *et al.*, 1985; Cormier *et al.*, 1990). Do and Cormier (1990, 1991b) have reported that an increase in osmotic potential brought about by raising the sucrose concentration results in enhanced accumulation of anthocyanin in grape cell cultures. However, the nature of the 'sucrose effect' in this and other culture systems has not been explained. Two possibilities will be considered:

(1) An osmotic effect due to the high concentration of the sucrose.

(2) An effect associated with the increased availability of a carbon source which slows down primary metabolism and promotes secondary metabolism.

4.5.1 The Effect of a Range of Sucrose Concentrations on Pigment Levels During the Batch Growth of Suspension Cultures

Several workers have investigated the effects of a range of sucrose concentrations on secondary metabolite production. In some instances there are reports of a positive correlation between sucrose concentration and the production of secondary metabolites. For example, the production of solasodine in callus cultures of *Solanum nigrum* (Bhatt *et al.*, 1983), lignin production in cultured sycamore cells (Carceller *et al.*, 1971) and anthocyanin production in *Vitis* suspension cultures (Yamakawa *et al.*, 1983). There are other reports of an optimum sucrose concentration for secondary metabolite production eg. 8% sucrose for the accumulation of alkaloids in dark grown suspension cultures of *Catharanthus roseus* (Knobloch and Berlin, 1980) and

5% sucrose for shikonin production in *Lithospermum erythrorhizon* callus cultures (Mizukami *et al.*, 1977).

In suspension cultures of *B.orellana* an increase in the sucrose concentration in the culture medium delayed cell division and growth of the cultures (see 3.5.3). The higher the sucrose concentration the longer the delay before an increase in cell number could be detected. Also, with higher sucrose concentrations, the period of pigment production was extended and greater pigment levels on a fresh weight basis were obtained. There also appeared to be a positive correlation between sucrose concentration and pigment production. These increases in the amount of pigment were at least partly due to an increase in the number of pigmented cells. At all sucrose concentrations the pattern of carotenoid accumulation was similar, with the production of pigment, once again, in the lag phase and early stages of growth. Pigment production ceased as cell division and growth progressed. Also, increasing the sucrose level in the culture medium did not reduce the viability of the cultures but there was evidence of plasmolysis of some cells in the MS12%S medium.

As the sucrose concentration in the culture medium is raised the osmolality also increases and the cells adapt to this increase in osmolality in order to remain viable and grow. It may be this adaptation to increased osmolality that delays the growth of the cultures. Table 4.2 shows the osmolality of control (3% sucrose) and MS12%S media after autoclaving. It also shows the osmolality of leaf cells and 14d. suspension culture cells grown on MS medium with 3% sucrose. The 14d. culture grown on 3% sucrose had an osmolality similar to the initial medium. The osmolality of MS12%S is similar to that of leaf cells. As mentioned previously cultured cells begin to plasmolyse in 12% sucrose. This may indicate that the osmotic potential at which cultured cells plasmolyse is a predetermined value similar to that in the plant. If this is so it would appear that cells in culture can withstand a lower osmolality than cells of the intact plant and that cultured cells have an osmolality similar to the medium in which they are grown. At all sucrose concentrations there was an initial rise in the osmolality of the culture medium following subculture. This is probably due to a rise in the concentration of reducing sugars since it is generally recognized that plant cell cultures hydrolyse sucrose to the reducing sugars, glucose and fructose, prior to their assimilation (Fowler and Stepan-Sarkissian, 1985; Stepan-Sarkissian and Fowler, 1986; Cresswell *et al.*, 1989).

Table 4.2

Osmolality values for various tissues and media of *B.orellana.*

Tissue/Medium	Osmolality (osmol.kg.$^{-1}$)
Leaves	0.579
14d. suspension cultures grown on control medium (3% sucrose)	0.251
Control Medium (3% sucrose)	0.234
MS12%S	0.562

As already suggested the delay in growth that occurs when the sucrose level is increased may be due to the cells adapting to a higher osmolality. Alternatively it may be due to an alteration in the C/N ratio. Increasing the carbon but not the nitrogen may inhibit growth and primary metabolism and therefore, enhance secondary metabolism. It has been suggested that when cells are dividing slowly common precursors appear to be diverted from primary to secondary pathways (Phillips and Henshaw, 1977; Lindsey and Yeoman, 1983). As the carbon level drops, as a result of secondary metabolism, it may reach a point where growth is again stimulated. However, from the measured sucrose levels in the culture medium this does not appear to happen (3.5.3).

Analysis of the pigments present in cultures grown on control (3% sucrose) and MS12%S showed that at the beginning of the culture period (d.3) both contained three carotenoid pigments, two of which were identified as zeaxanthin and β-carotene (see 3.5.4). This is consistent with reports for carrot cell cultures where both zeaxanthin and β-carotene have been found (Shimizu *et al.*, 1979). At the end of the culture period (d.24) the three pigments were again present but the cultures on MS12%S contained more of these pigments and three additional pigments. The three additional pigments on MS12%S were not identified but they were similar to pigments found in the plant. In general, the cultures contained less carotenoid

pigment than the plant but despite the similarities between plant and cultures, bixin was not present in the suspension cultures. These findings are not surprising since a regular problem encountered with plant tissue cultures is that with few exceptions the cultures accumulate lower levels of secondary metabolites than the plant (Fowler, 1986; Collin, 1987) and frequently the substances produced are different from those found in the intact plant.

As already mentioned there could be at least two explanations for the 'sucrose effect'. The increased availability of carbon and/or the osmotic effect of sucrose. In the literature there are reports of sucrose inhibiting chlorophyll synthesis, for example, in carrot callus cultures (Edelman and Hanson, 1971) and spinach suspension cultures (Dalton and Street, 1977). Edelman and Hanson (1971) found that sucrose caused both a reduction in chloroplast number per cell and suppressed lamellar development in plastids in carrot callus cultures. Further, it has been found that transcription of seven maize photosynthetic gene promoters is repressed by sucrose, a photosynthetic end product (Sheen, 1990). Chlorophyll formation as well as structural and functional development of chloroplasts is also reported to be influenced by water stress (see Sundqvist *et al.*, 1980). Whatever the effect of sucrose it does appear to distort chloroplast development so it may also modify the development of other plastids. Chromoplasts can develop from chloroplasts eg. in fruit ripening (Kirk and Tilney-Bassett, 1978), therefore perhaps the presence of sucrose in the culture medium of *B.orellana* inhibits chloroplast development and so stimulates chromoplast formation. Indeed, the interconversion of chloroplasts and chromoplasts in the epicarp of citrus fruits has been shown to be reversible and largely dependant on the availability of nitrogen and abundance of sugars (Huff, 1983, 1984). The accumulation of sugars in the epicarp tended to favour the presence of chromoplasts.

The results reported in this study are consistent with those of Cormier *et al.* (1990) who found that the growth of grape cell cultures was reduced and the lag phase prolonged when the sucrose level in the culture medium was raised. They also found that the accumulation of anthocyanin increased and maximum pigment production occurred during the prolonged lag phase. In this instance it was found that osmotic stress due to the increased sucrose concentration in the culture medium was involved in prolonging the lag phase and raising anthocyanin production (Do and Cormier, 1990, 1991b). An osmotic effect may also be responsible for the increased carotenoid levels in this study.

4.5.2 The Osmotic Effect of Sucrose Concentration on Pigment Formation

In the literature there are several reports of an increase in sucrose concentration promoting secondary metabolism in culture and in some instances osmotic stress has been implicated (Do and Cormier, 1990, 1991b). Further, water stress has been shown to favour β-carotene synthesis in leaves of *Digitalis lanata* (Stuhlfauth *et al.*, 1990).

When the control medium was supplemented with mannitol (MS3%S+M) to adjust the osmolality to a value similar to the MS12%S medium the growth of *B.orellana* suspension cultures was decreased and the pigment per g. fresh weight increased (see 3.6), indicating that the increase in pigment production at raised sucrose concentrations was due to an osmotic effect. Some sugars breakdown during autoclaving (see Schenk *et al.*, 1991) and this could account for the differences in the osmolality of the MS3%S+M and MS12%S media. These differences in the media after autoclaving may be the reason why the biomass decrease and pigment increase were smaller with MS3%S+M than with MS12%S. The higher osmolality with MS12%S possibly meant that growth was delayed longer and more pigment was produced giving values different to MS3%S+M.

Less sucrose was utilised when the culture medium contained mannitol, presumably because there was less growth. Here it would seem that mannitol regulates the utilization of sucrose. The viability of the cultures was not affected when the osmolality of the culture medium was increased and as previously reported for other treatments the rise in pigment was partly due to an increase in the number of pigmented cells. It was also noted that increasing the osmolality of the medium resulted in a decrease in cell size (see Table 4.3). This observation is in agreement with the findings of Kimball *et al.* (1975) who showed that cell size decreases as the concentration of sucrose, glucose, mannitol or sorbitol is increased. They suggested that the reduction in cell size was due to an osmotic effect. Osmotic stress has also been reported to affect the production of some secondary metabolites. For example, alkaloid production in cell cultures of *Catharanthus roseus* (Rudge and Morris, 1986) and accumulation of anthocyanin in *Vitis vinifera* cell cultures (Do and Cormier, 1990, 1991b).

Table 4.3

Changes in fresh weight per cell in suspension cultures of *B.orellana* during batch growth. Each value is the mean of three replicates ± s.e.

Time (Days)	Fresh Weight per Cell (μg.)		
	Control (3%S)	**MS12%S**	**MS3%S+Mannitol**
0	0.115 ± 0.002	0.115 ± 0.002	0.115 ± 0.002
3	0.102 ± 0.003	0.092 ± 0.004 *	0.089 ± 0.003 **
24	0.174 ± 0.014	0.120 ± 0.005 **	0.128 ± 0.003 **

(significant from the control at *P=0.1 and **P=0.05)

From this study it can be concluded that increasing the osmolality of the culture medium stimulates carotenoid pigment production in suspension cultures of *B.orellana*. This treatment delays cell division which lengthens the lag phase and in turn stimulates pigment production. The fact that MS3%S+M gave similar results to MS12%S shows that an osmotic effect must be involved when the sucrose concentration in the medium is raised. Mannitol is a non-assimilable sugar so the carbon available in MS3%S+M was the same as the control and the main difference between these two media was different osmolalities. Therefore, these results strongly indicate that increased available carbon in media with raised sucrose levels was not stimulating pigment production and the 'sucrose effect' appears to be due to the increased osmolality of the culture medium. The nature of this osmotic effect is still not understood. It may be stimulating plastid development and pigment production directly or it may have an indirect effect by inhibiting growth.

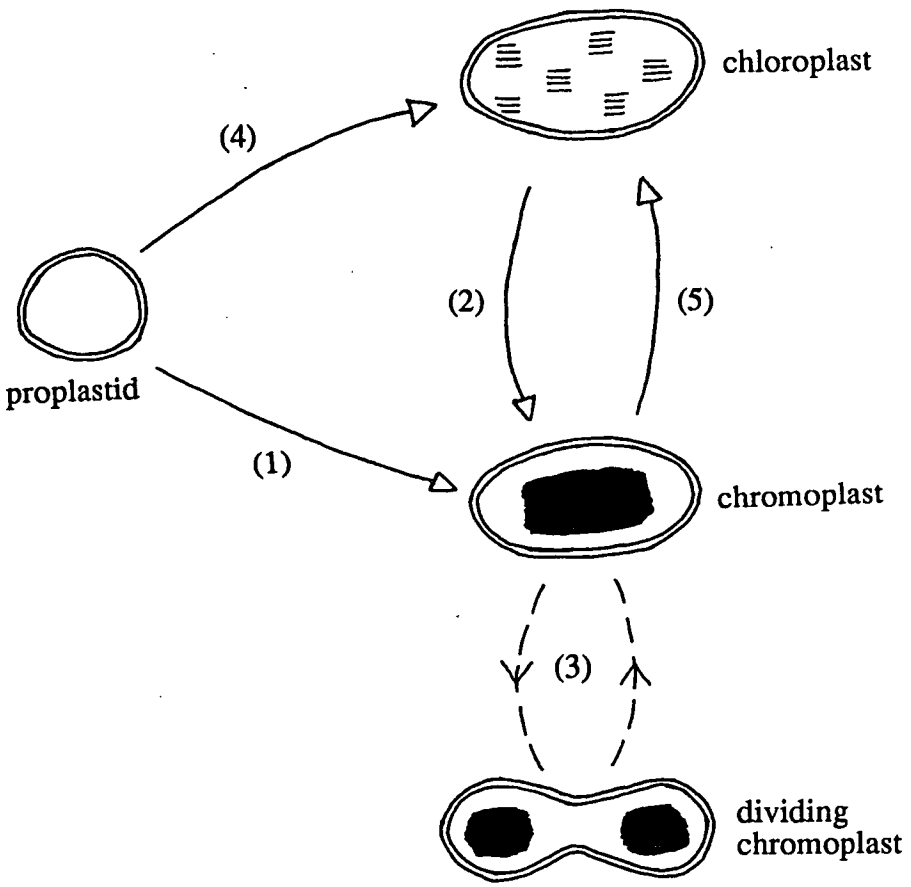
When the osmolality of the culture medium was raised, either with sucrose or mannitol, an increase in the proportion of pigmented cells occurred indicating that there was a rise in the number of pigmented cells and therefore, the number of

chromoplasts must have increased. An increase in the number of chromoplasts could occur via several routes some of which have already been mentioned. These are summarized in Fig. 4.1. Route (1) shows the conversion of proplastids into chromoplasts (see Kirk and Tilney-Bassett, 1978). Route (2) shows the conversion of chloroplasts into chromoplasts which has been shown to occur in ripening fruit (Kirk and Tilney-Bassett, 1978). Such a conversion was found to be dependent on carbon and nitrogen levels in the epicarp of citrus fruit (Huff, 1983, 1984). The third route (3) shows the division of chromoplasts. This has not been reported, however, division of chloroplasts and proplastids is known to occur (see Possingham, 1980). Stimulation of routes (1), (2) and (3) could lead to a rise in chromoplast number and, therefore, increase pigment levels. Route (4) shows the conversion of proplastids into chloroplasts (see Kirk and Tilney-Bassett, 1978; Mullet, 1988) and route (5) shows the conversion of chromoplasts into chloroplasts (see Huff, 1983, 1984). Inhibition of routes (4) and (5) could also be involved in raising the chromoplast number.

Overall it can be concluded that both osmotic and nutrient stress (nitrogen depletion) can result in increased pigment production in *B.orellana* cultures. It would appear that for osmotic stress an increase in chromoplast number is probably involved. The amount of pigment per cell and chromoplast number were not measured so the possibility of changes in pigment per cell occurring, either due to increased numbers of chromoplasts per cell or increased pigment per chromoplast, cannot be ruled out. It may be that nitrogen depletion and osmotic stress are having similar effects on pigment production and an increase in chromoplast number would therefore probably be involved. Nitrogen has been shown to be important in the interconversion of chloroplasts and chromoplasts (routes (2) and (5) in Fig. 4.1) in the epicarp of citrus fruit (Huff, 1983, 1984). It is not known whether osmotic stress is affecting pigment production directly or if it is an indirect effect through delayed growth. However, the evidence for nitrogen depletion tends to favour a direct effect since the pigment increased in the lag phase but the length of the lag phase was not altered.

Figure 4.1

A diagram showing the possible routes involved in altering the chromoplast number and the influence of osmotic stress and nitrogen depletion on those routes such that an overall increase in the number of chromoplasts is obtained.



(1), (2) and (3) are possibly stimulated by osmotic stress or nitrogen depletion.

(4) and (5) are possibly inhibited by osmotic stress or nitrogen depletion.

FUTURE WORK

The results obtained in this investigation have shown that osmotic stress can increase pigment production in cultures of *B.orellana*. Future investigations could involve a more detailed examination of this effect to determine its nature ie. how it affects cell development and metabolism. The principle components for this future work are outlined below:

(1) Investigation at the Cellular Level

(a) Examination of changes in plastid number and development during culture growth using electron microscopy. This should show if developmental changes are related to pigment production.

(b) The yield of a metabolite from plant tissue culture depends on the proportion of cells which are accumulating the product and the amount of product within these productive cells. Determination of changes in the number of cells accumulating pigment and the pigment per cell would help to decide a method(s) of increasing the yield. The proportion of pigmented cells can be an important determinant in the amount of pigment produced in a culture (3.5.2, 3.5.3 and 3.6). Further measurements should be made using microdensitometric techniques, similar to those used by Hall and Yeoman (1986a), to determine pigment levels per cell. Such measurements would also give an indication of the degree of heterogeneity within the productive cell population.

Both these studies should be conducted under different osmotic conditions to examine the correlation between cell development and pigment level.

(2) Investigation of Whether the Osmotic Effect has a Direct or Indirect Effect on Pigment Production

To determine whether growth is directly coupled to pigment production the growth of the cultures could be delayed by lowering the temperature (see M^{ac}Carthy and Stumpf, 1980) or using a smaller inoculum (see Gamborg and Shyluk, 1981). This should give an indication of whether growth is directly involved in pigment production. It could also show if the osmotic effect was affecting pigment production directly or if it was an indirect effect through delayed growth.

(3) Investigation of Substrate Utilization

Substrate utilization could be studied by investigating the fate of radioactively labelled precursors eg. universally labelled sucrose or mevalonic acid (MVA). This could determine whether the added carbon source or carbon stored in the cell is utilized for carotenoid production. Also by measuring label in different compounds, eg. sterols and carotenoids, it could provide information on the extent of primary and secondary pathways. Such studies should be carried out under different osmotic conditions to allow comparisons to be made and so give further insight into the nature of the osmotic effect.

(4) Investigation of Bixin Production

To investigate bixin production radioactively labelled precursors of the general carotenoid pathway could be used eg. MVA. The precursors would be added to plant tissue such as roots, which are known to contain bixin (3.1.3), since bixin was not produced in cell cultures. Addition of such precursors to plant tissue and measurement of label in bixin would indicate if the labelled compound is in the pathway and also show if osmotic stress stimulates synthesis. It may be that the enzymes and/or substrates for bixin production are present in culture but are not available because of compartmentation. Addition of radioactively labelled precursors to a cell free extract of cultured cells with subsequent measurement of label in bixin should indicate if the enzymes and/or substrates are present.

Such investigations should give a better understanding of the nature of the osmotic effect in suspension cultures and its effect on bixin production in particular. They may also give some insight as to why bixin production was not stimulated in suspension cultures.

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THE PRODUCTION OF BIXIN BY CULTURES OF BIXA ORELLANA

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The orange-red carotenoid bixin (E160b) which is used as a food colouring, is obtained commercially from the seeds of Bixa orellana. The production of bixin by plant tissue culture was investigated. Both callus and suspension cultures of B. orellana were established. Pigmented cells were found in these cultures, however, the pigment disappears as the culture ages. Bixin can be found in many parts of the plant, e.g. leaves, root, stem. Investigation of the pigment level during callus initiation from root tissue has shown that the amount of pigment decreases initially but the level rises later. The distribution of pigment between the root tissue and newly initiated callus was investigated. In suspension cultures the level of pigment was found to increase during the lag phase of the growth curve then decreased as growth continued.